FORM PTO-1390 REV. 2/01T — U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
04853.0087	
U.S. APPLICATION NO. (If known, see 37CFR1.5)	10/019785

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/04523	July 6, 2000	July 6, 1999

TITLE OF INVENTION
THERAPEUTIC AGENT FOR DRUG-RESISTANT HYPERCALCEMIA

APPLICANT(S) FOR DO/EO/US
Hidemi SAITO, Toshiaki TSUNENARI, and Etsuro ONUMA

Applicant(s) herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c)(2)).
   - a. ☐ is attached hereto (required only if not communicated by the International Bureau.
   - b. ☒ has been communicated by the International Bureau.
   - c. ☐ is not required, as the application was filed with the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
   - a. ☒ is attached hereto.
   - b. ☐ has been previously submitted under 35 U.S.C. 154 (d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)).
   - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ have been communicated by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ Information Disclosure Statement under 37 CFR 1.97 and 1.98
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A Substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154 (d)(4).
19. ☐ A second copy of the English language translation of the international application 35 U.S.C. 154 (d)(4).
20. ☒ Other items or information:
    - a. ☒ Copy of cover page of International Publication No. WO 01/02012
    - b. ☒ Sequence Listing in written form consisting of 47 pages
    - c. ☒ Deposit of Microorganisms for Accession Nos. FERM BP-5627/-5628/-5629/-5630/-5631, and English Translations thereof

U.S. APPLICATION NO. (If known, see 37CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/019785	PCT/JP00/04523	04853.0087

21. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTOand all claims satisfied provisions of PCT Article 33 (1)-(4) **$100.00**

CALCULATIONS PTO USE ONLY

ENTER APPROPRIATE BASIC FEE AMOUNT =					$890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).					$	
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE		
Total Claims	16	- 20 =		x $18.00	$	
Independent Claims	1	-3 =		x $84.00	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+$280.00	$280.00	
TOTAL OF THE ABOVE CALCULATIONS =					$1170.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.					$	
SUBTOTAL =					$1170.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest priority date (37 CFR 1.492(f)).					$	
TOTAL NATIONAL FEE =					1170.00	
Fee for recording the enclosed assignment (37 CFR 1.21 (h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property. +					$40.00	
TOTAL FEES ENCLOSED =					$1210.00	
					Amount to be refunded:	$
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a. ☒ A check in the amount of $ 1210.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-0916. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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DATED: January 4, 2002

SIGNATURE

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Attorney Docket No. 04853.0087
Customer Number 22,852

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. National Phase Application of: PCT/JP00/04523	)	Group Art Unit:
Serial No.: Not Yet Assigned	)	Examiner:
Filed: January 4, 2002	)	
For: THERAPEUTIC AGENT FOR DRUG-RESISTANT HYPERCALCEMIA	)	

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

STATEMENT

The information recorded in computer readable form (attached diskette) is identical to the written sequence listing.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.

Dated: January 4, 2002

By _____
Ernest F. Chapman
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EFC/FPD/gah
Enclosures

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DESCRIPTION

THERAPEUTIC AGENT FOR DRUG-RESISTANT HYPERCALCEMIA

TECHNICAL FIELD

The present invention relates to a therapeutic agent for drug-resistant hypercalcemia, which comprises, as an active ingredient, a substance capable of inhibiting the binding between parathyroid hormone related protein (PTHrP) and a receptor.

BACKGROUND ART

In drug therapy (i.e., pharmacotherapy) for humoral hypercalcemia of malignancy (HHM), a supplemental infusion/a loop diuretic or a bone resorption-inhibiting agent (e.g., bisphosphonate, or calcitonin) is administered. Current trend of the drug therapy is an administration of calcitonin or a bisphosphonate alone and combination of calcitonin and a bisphosphonate.

The calcitonin administration has a quite rapid onset of action, and the therapeutic effect is expressed within several hours after the administration. Calcitonin is effective primarily due to its bone resorption-inhibitory action. For application, a calcitonin may be injected to a patient intramuscularly or intravenously twice a day (in morning and evening) in a dose of 40 to 80 units.

A bisphosphonate is a compound which is effective due to its very potent bone resorption-inhibitory action. It has a slow onset of action which requires three to four days until the therapeutic effect is expressed. Then, a combination therapy consisting of a calcitonin and a bisphosphonate (which is not rapid acting but has a potent bone resorption-inhibiting action) is also employed.

Unfortunately, a bisphosphonate and a calcitonin may have the following limits in use.

(1) Bisphosphonate:

· Although it can inhibit the bone resorption, it cannot improve the calcium excretion in the kidney.

· It has a slow onset of action which requires three to four days until the therapeutic

effect is expressed.

· It is poorly effective in patients with high blood levels of PTHrP (12 pmol/L or higher), and has a shorter duration of action.

· It is attenuated in its action when administered repeatedly. For repeated administration, it is required that it be administered at intervals of at least one week for the purpose of confirming the response induced by the initial administration.

· It may sometimes cause hypocalcemia.

(2) Calcitonin:

Its effect on the reduction of calcium levels reaches maximum within 12 to 24 hours after the administration. When a repeated administration is performed thereafter, however, the effect may show a re-ascent within 4 to 5 days after the administration, which is known as the so-called "escape phenomenon".

For the reasons set forth above, these drugs are limited in their clinical use due to the attenuated efficacy caused by repeated administration or the development of resistance to the drugs. Moreover, these drugs are not so satisfactory for improvement in QOL of HHM patients.

DISCLOSURE OF THE INVENTION

The object of the present invention is to provide a therapeutic agent for drug-resistant hypercalcemia, which comprises, as an active ingredient, a substance capable of inhibiting the binding between parathyroid hormone related protein (PTHrP) and a receptor thereof.

The present inventors have made extensive and intensive studies for overcoming the drawbacks set forth above. As a result, the inventors have found that a substance capable of inhibiting the binding between PTHrP and a receptor thereof could ameliorate hypercalcemia resistant to existing therapeutic agent for hypercalcemia such as bone resorption-inhibiting agents. This finding led the accomplishment of the invention.

Accordingly, the present invention provides a therapeutic agent for drug-resistant hypercalcemia comprising, as an active ingredient, a substance capable of inhibiting the

binding between PTHrP and a receptor thereof. In this regard, the drug-resistant hypercalcemia may be resistant to a therapeutic agent for hypercalcemia other than a substance capable of inhibiting the binding between PTHrP and a receptor thereof. The substance may be an antagonist for the PTHrP receptor, an anti-PTHrP antibody, which may be monoclonal or polyclonal, preferably of monoclonal type. The monoclonal antibody may be a humanized or chimeric monoclonal antibody or a human antibody, or a fragment of the antibody and/or a modified form of the fragment. The humanized antibody may be humanized #23-57-137-1 antibody. The drug-resistant hypercalcemia may be caused by cancer.

The "drug" in the "drug-resistant hypercalcemia" may be any drug or agent as long as it is therapeutically effective on hypercalcemia, such as a bone resorption-inhibiting agent, a calcium excretion-promoting agent, an agent for inhibiting gastrointestinal absorption of calcium and a loop diuretic.

The "drug-resistant hypercalcemia" as used herein refers to a class of hypercalcemia which exhibits resistance to a drug having a therapeutic efficacy on hypercalcemia when the drug is administered repeatedly, such as hypercalcemia resistant to a bone resorption-inhibiting agent, a calcium excretion-promoting agent, an agent for inhibiting gastrointestinal absorption of calcium and a loop diuretic, preferably a bone resorption-inhibiting agent.

The bone resorption-inhibiting agent may include a bisphosphonate, calcitonin and so on.

The bisphosphonate may include, for example, alendronate, pamidronate and incadronate.

The calcitonin may include, for example, calcitonin, elcatonin and salmon calcitonin.

The calcium excretion-promoting agent may include calcitonin and a loop diuretic.

The agent for inhibiting gastrointestinal absorption of calcium may include a glucocorticoid and an inorganic phosphate.

The loop diuretic may include furosemide.

The osteoclast-inhibiting agent may include an anti-cancer agent, such as actinomycin D, cisplatin and mithramycin.

Hereinbelow, the present invention will be illustrated in detail.

This specification includes part or all of the contents as disclosed in the specification and/or drawings of Japanese Patent Application No. 11-192270, which is a priority document of the present appliction.

The present invention relates to a therapeutic agent for drug-resistant hypercalcemia, which comprises, as an active ingredient, a substance capable of inhibiting the binding between parathyroid hormone related protein (hereinafter, referred to as "PTHrP") and a receptor thereof (hereinafter, referred to as "PTHrP receptor").

As used herein, the term "PTHrP receptor" refers to any receptor that can bind to PTHrP (such as those as described in Japanese National Phase Laid-open Publication No. 6-506598), regardless of whether or not the PTHrP receptor is present on a target organ (e.g., bone, kidney).

As used herein, the term "a substance capable of inhibiting the binding between PTHrP and a receptor thereof (a PTHrP receptor)" refers to any substance that can bind to PTHrP to prevent the binding of the PTHrP to a PTHrP receptor, such as an anti-PTHrP antibody; any substance that can bind to a PTHrP receptor to prevent the binding of the PTHrP receptor to PTHrP, such as an antagonist against a PTHrP receptor (a PTHrP antagonist), specifically a peptide having replacement or deletion of at least one amino acid residue in the PTHrP peptide or a partial sequence of the PTHrP peptide; or a combination thereof.

The anti-PTHrP antibody includes those of any known types, such as a humanized antibody, a human antibody (WO 96/33735) or a chimeric antibody (Japanese Patent Application Laid-open No. 4-228089), and an antibody produced from hybridoma #23-57-137-1 (i.e., #23-57-137-1 antibody). The antibody may be of polyclonal type or monoclonal type, but preferably of monoclonal type. The PTHrP antagonist includes, but is not limited to, a polypeptide or a low molecular weight substance. An example of the PTHrP antagonist is a substance that binds to a PTHrP receptor in an antagonistic manner against PTHrP, such as a PTHrP (7-34), PTHrP (8-34), PTHrP (9-34), PTHrP (10-34), or mutants thereof which have replacement, deletion or addition of at least one amino acid residue. The additional examples thereof include polypeptides having an antagonistic activity against PTHrP as described in Japanese Patent Application Laid-open No. 7-165790; Japanese National Phase Laid-open No.

5-509098; Peptides (UNITED STATES), 1995, 16(6) 1031-1037; and Biochemistry (UNITED STATES) Apr. 281992, 31(16) 4026-4033. These polypeptides may have deletion, replacement, addition or insertion of at least one amino acid residue, as long as they can have an equivalent level of PTHrP antagonistic activity, which are also encompassed in the PTHrP antagonists of the present invention.

In the present invention, as an example of the "substance capable of inhibiting the binding between PTHrP and a PTHrP receptor", an anti-PTHrP antibody will be explained below.

1. Anti-PTHrP antibody

The anti-PTHrP antibody used in the present invention may be any one as long as it can exhibit a therapeutic effect on drug-resistant hypercalcemia, regardless of its source, type (monoclonal or polyclonal) and configuration.

The anti-PTHrP antibody used in the present invention can be produced by any known method as a polyclonal or monoclonal antibody. Preferably, the anti-PTHrP antibody is a monoclonal antibody derived from a mammal. The mammal-derived monoclonal antibody includes those produced from a hybridoma and those produced by a genetic engineering technique from a host transformed with a recombinant expression vector carrying a gene for the antibody. The antibody can bind to PTHrP to prevent the binding of the PTHrP to a PTH/PTHrP receptor, thus blocking the signal transduction of the PTHrP and consequently inhibiting the biological activity of the PTHrP.

A specific example of such antibody is #23-57-137-1 antibody which can be produced with a hybridoma clone #23-57-137-1.

The hybridoma clone #23-57-137-1 has been designated "mouse-mouse hybridoma #23-57-137-1" and deposited under the terms of the Budapest Treaty on August 15, 1996 at the National Institute of Bioscience and Human-technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) under the accession No. FERM BP-5631.

2. Antibody-producing hybridoma

A monoclonal antibody-producing hybridoma can be produced as follows. That is,

PTHrP is used as an antigen for immunization in accordance with a conventional immunization method. The resulting immunocytes are fused to known parent cells by a conventional cell fusion method, and monoclonal antibody-producing cells are screened from the fused cells by a conventional screening method.

First, a human PTHrP, which is used as an sensitizing antigen for producing the antibody, is prepared by expressing the PTHrP gene/amino acid sequence disclosed in Suva, L. J. et al., Science (1987) 237, 893. A nucleotide sequence encoding the PTHrP is inserted into a known expression vector, and a suitable host cell is transformed with the expression vector. The PTHrP protein is then isolated and purified from the transformed host cell or from a culture supernatant of the transformed host cell by any known method.

Then, the purified PTHrP protein is used as a sensitizing antigen. Alternatively, a 34-amino acid peptide of the N-terminal region of the PTHrP may be chemically synthesized as the sensitizing antigen.

The mammal to be immunized with the sensitizing antigen is not particularly limited. However, the mammal is preferably selected taking into consideration of compatibility with the patent cell used for cell fusion. Generally, a rodent (e.g., mouse, rat, hamster), rabbit or monkey may be used.

The immunization of the mammal with the sensitizing antigen can be performed in accordance with any known method, for example, by injecting the sensitizing antigen to a mammal intraperitoneally or subcutaneously. More specifically, the sensitizing antigen is properly diluted with or suspended to phosphate-buffered saline (PBS) or physiological saline, the resulting dilution or suspension is then mixed with an appropriate amount of a conventional adjuvant (e.g., Freund's complete adjuvant) to give an emulsion. The emulsion is injected to a mammal several times at intervals of 4 to 21 days. For the immunization, the sensitizing antigen may be attached to a suitable carrier.

After the immunization, the serum antibody level is checked. When the serum antibody level is confirmed to reach a desired level, immunocytes are isolated from the mammal and then subjected to cell fusion. A preferable immunocyte is a spleen cell.

The parent cell used for the cell fusion (i.e., the counterpart of the cell fusion with the

immunocyte) is a myeloma cell derived from a mammal. The myeloma cell is of any known cell line, and, for example, P3 (P3x63Ag8.653) (J. Immnol. (1979) 123, 1548-1550), P3x63Ag8U.1 (Current Topics in Microbiology and Immunology (1978) 81, 1-7), NS-1 (Kohler, G. and Milstein, C. Eur. J. Immunol. (1976) 6, 511-519), MPC-11 (Margulies, D. H. et al., Cell (1976) 8, 405-415), SP2/0 (Shulman, M. et al., Nature (1978) 276, 269-270), FO (de St. Groth, S. F. et al., J. Immunol. Methods (1980) 35, 1-21), S194 (Trowbridge, I. S., J. Exp. Med. (1978) 148, 313-323) or R210 (Galfre, G. et al., Nature (1979) 277, 131-133).

Cell fusion of the immunocyte to the myeloma cell is basically performed in accordance with any known method, such as the method of Milstein et al. (Kohler, G. and Milstein, C., Methods Enzymol. (1981) 73, 3-46).

More specifically, the cell fusion is performed, for example, in a conventional nutrient culture medium in the presence of a cell fusion promoter. The cell fusion promoter may be polyethylene glycol (PEG) or a Sendai virus (hemagglutinating virus of Japan; HVJ). If desired, for the purpose of improving the fusion efficiency, an additive such as dimethyl sulfoxide may be incorporated.

The ratio between the immunocytes and the myeloma cells for the cell fusion may be any one. For example, the immunocytes are used in the amount 1-10 times larger than the myeloma cells. The culture medium used for the cell fusion is, for example, RPMI 1640 medium or MEM medium suitable for the growth of the above-mentioned myeloma cell lines, or other medium conventionally used for the culture of such cell lines. If desired, a serum supplement, such as feral calf serum (FCS), may be added to the culture medium.

The cell fusion is performed by fully mixing given amounts of the immunocytes and the myeloma cells in the culture medium, adding a PEG solution (e.g., mean molecular weight: about 1000-6000) (which has been previously warmed to about 37° C) to the mixture usually to a concentration of 30-60% (w/v), and then mixing the resulting solution, thereby producing the desired fusion cells (i.e., hybridomas). Subsequently, an appropriate culture medium is added to the culture solution successively, and centrifuged to remove the supernatant. This procedure is repeated several times to remove the cell fusion promoter or the like that are undesirable for the growth of the hybridomas, from the culture medium.

The obtained hybridomas can be selected by culturing in a conventional selective medium, such as hypoxanthine-aminopterin-thymidine (HAT) medium. The culturing of the hybridomas in HAT medium is performed for the time of period enough to cause the death of the cells other than the desired hybridomas (i.e., cells that fail to fuse), usually for several days to several weeks. Subsequently, conventional limiting dilution method is performed for screening and mono-cloning of the hybridomas that are secreting the desired antibody.

As a method other than preparing the hybridomas by immunizing a non-human mammal with the antigen as described above, a human lymphocyte may be sensitized with PTHrP in vitro, and then subjected the sensitized lymphocyte to cell fusion to a human-derived myeloma cell capable of infinite growth, thereby producing a human antibody having a binding activity against the PTHrP (Japanese Patent Publication No. 1-59878). Alternatively, a human antibody against PTHrP may be prepared by injecting PTHrP as an antigen to a transgenic animal that has the entire repertories of human antibody genes to produce an anti-PTHrP antibody-producing cell, and then immortalizing the cells, thus producing the human antibody from the immortalized cell (International Patent Publication Nos. WO 94/25585, WO 93/12227, WO 92/03918 and WO 94/02602).

The monoclonal antibody-producing hybridoma prepared as above can be subcultured in a conventional culture medium and stored under liquid nitrogen for a long time of period.

For the production of a monoclonal antibody from the hybridoma, a method may be employed that involves culturing the hybridoma in accordance with a conventional technique and collecting the monoclonal antibody from the culture supernatant, or that involves injecting the hybridoma to a mammal compatible with the hybridoma to grow the hybridoma in the mammal and collecting the hybridoma from the ascites of the mammal. The former method is suitable for producing the antibody in high purity, while the latter method is suitable for producing the antibody in a large amount.

3. Recombinant antibody

In the present invention, a recombinant-type monoclonal antibody may be used, which can be produced by cloning an antibody gene from the hybridoma, integrating the antibody

gene into a suitable vector, introducing the vector into a host, and then producing the antibody from the host according to a conventional genetic recombination technique (see, for example, Vandamme, A. M. et al., Eur. J. Biochem. (1990) 192, 767-775, 1990)

Specifically, mRNA encoding variable (V) region of an anti-PTHrP antibody is isolated from the anti-PTHrP antibody-producing hybridoma. The isolation of the mRNA is performed by preparing a total RNA by any known method, such as guanidium ultracentrifugation method (Chirgwin, J. M. et al., Biochemistry (1979) 18, 5294-5299) and AGPC method (Chomczynski, P. et al., Anal. Biochem. (1987) 162, 156-159), and then producing the desired mRNA from the total RNA using mRNA Purification Kit (Pharmacia) or the like. Alternatively, the mRNA may also be prepared directly using QuickPrep mRNA Purification Kit (Pharmacia).

Next, cDNA for the antibody V-region is synthesized from the mRNA with a reverse transcriptase. The synthesis of the cDNA is performed using AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Corporation) or the like. The cDNA may also be synthesized and amplified by 5'-RACE method (Frohman, M.A. et al., Proc. Natl. Acad. Sci. USA (1988) 85, 8998-9002; Belyavsky, A. et al., Nucleic Acids Res. (1989) 17, 2919-2932) using 5'-Ampli FINDER RACE Kit (CLONETECH) in combination with PCR method, or the like.

A DNA fragment of interest is isolated and purified from the resulting PCR product and then ligated to a vector DNA to obtain a recombinant vector. The recombinant vector is introduced into a host such as E. coli, and a colony containing a desired recombinant vector is selected. The nucleotide sequence of the DNA of interest in the recombinant vector is confirmed by, for example, dideoxynucleotide chain termination method.

Once DNA encoding the anti-PTHrP antibody V-region is obtained, the DNA is integrated into an expression vector containing a DNA encoding a desired antibody constant (C) region.

For the production of the anti-PTHrP antibody used in the present invention, the antibody gene is integrated into an expression vector so that the antibody gene can be expressed under the control of expression control regions (e.g., enhancer, promoter). A host cell is transformed with the expression vector to express the antibody.

In the expression of the antibody gene, a DNA encoding heavy (H) chain and a DNA encoding light (L) chain of the antibody may be integrated into separate expression vectors, and then a host cell is co-transformed with the resulting recombinant expression vectors. Alternatively, both the DNA encoding H-chain and the DNA encoding L-chain of the antibody may be integrated together into a single expression vector, and then a host cell may be transformed with the resulting recombinant expression vector (WO 94/11523).

For the production of the recombinant antibody, besides the above-mentioned host cells, a transgenic animal may also be used as a host. For example, the antibody gene is inserted into a predetermined site of a gene encoding a protein inherently produced in the milk of an animal (e.g., goat β -casein) to obtain a fusion gene. A DNA fragment containing the antibody gene-introduced fusion gene is injected into an embryo of a goat, and the embryo is then introduced into a female goat. The female goat having the embryo therein bears a transgenic goat. The antibody of interest is secreted in the milk from the transgenic goat or a progeny thereof. For the purpose of increasing the amount of the antibody-containing milk from the transgenic goat, an appropriate hormone may be administered to the transgenic goat (Ebert, K.M. et al., Bio/Technology (1994) 12, 699-702).

4. Modified antibody

In the present invention, for the purpose of reducing the heterogenisity against a human body or the like, an artificially modified recombinant antibody may be used, such as a chimeric antibody and a humanized antibody. These modified antibodies can be prepared by the following known methods.

A chimeric antibody usable in the present invention can be prepared by ligating the DNA encoding the antibody V-region prepared as set forth above to a DNA encoding a human antibody C-region, integrating the ligation product into an expression vector, and introducing the resulting recombinant expression vector into a host to produce the chimeric antibody.

A humanized antibody is also referred to as a "reshaped human antibody", in which the complementarity determining regions (CDRs) of an antibody of a non-human mammal (e.g., a mouse) are grafted to those of a human antibody. The general genetic recombination

procedures for producing such humanized antibody are also known (EP 125023; WO 96/02576).

Specifically, a DNA sequence in which mouse antibody CDRs are ligated through framework regions (FRs) of a human antibody is amplified by PCR method using several oligonucleotides as primers which have been designed to have regions overlapping to the terminal regions of the CDRs and the FRs. The resulting DNA is ligated to a DNA encoding a human antibody C-region, and the ligation product is integrated into an expression vector. The resulting recombinant expression vector is introduced into a host, thereby producing the humanized antibody (EP 239044, WO 96/02576).

The FRs of the human antibody ligated through the CDRs are selected so that the CDRs can form a suitable antigen binding site. If necessary, an amino acid(s) in the FRs of the antibody V-region may be replaced so that the CDRs of the reshaped human antibody can form a suitable antigen binding site (Sato, K. et al., Cancer Res. (1993) 53, 851-856).

The C-region of the chimeric or humanized antibody may be any human antibody C-region, such as C γ 1, C γ 2, C γ 3 or C γ 4 for the H-chain, and C κ or C λ for the L-chain. The human antibody C-region may be modified for the purpose of improving the stable production of the antibody.

The chimeric antibody is composed of V-regions derived from a non-human mammalian antibody and C-regions derived from a human antibody. The humanized antibody is composed of CDRs derived from a non-human mammalian antibody and FRs and C-regions derived from a human antibody. The humanized antibody is useful as an active ingredient for the therapeutic agent of the present invention, because the antigenicity of the antibody against a human body is reduced.

A specific example of the humanized antibody usable in the present invention is humanized #23-57-137-1 antibody; in which the CDRs are derived from mouse-derived #23-57-137-1 antibody; the L-chain is composed of the CDRs ligated through three FRs (FR1, FR2 and FR3) derived from human antibody HSU 03868 (GEN-BANK, Deftos, M. et al., Scand. J. Immunol., 39, 95-103, 1994) and a FR (FR4) derived from human antibody S25755 (NBRF-PDB); and the H-chain is composed of the CDRs ligated through FRs derived from human

antibody S31679 (NBRF-PDB, Cuisinier, A. M. et al., Eur. J. Immunol. 23, 110-118, 1993) in which a part of the amino acid residues in the FRs is replaced so that the reshaped humanized antibody can exhibit an antigen-binding activity.

The E. coli strains containing the plasmids having DNAs encoding the H-chain and the L-chain of the humanized #23-57-137-1 antibody, respectively, are designated Escherichia coli JM109 (hMBC1HcDNA/pUC19) (for H-chain) and Escherichia coli JM109 (hMBC1Lq λ /pUC19) (for L-chain), respectively. These strains have been deposited under the terms of the Budapest Treaty on August 15, 1996 at the National Institute of Bioscience and Human-technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan), under the accession No. FERM BP-5629 for Escherichia coli JM109 (hMBC1HcDNA/pUC19), and under the accession No. FERM BP-5630 for Escherichia coli JM109 (hMBC1Lq λ /pUC19).

5. Antibody variants

The antibody used in the present invention may be a fragment thereof or a modified form of the fragment, as long as it can bind to PTHrP and inhibit the activity of the PTHrP. For example, the fragment of the antibody includes Fab, $F(ab')_2$, Fv, or a single chain Fv (scFv) composed of a H-chain Fv fragment and a L-chain Fv fragment linked together through a suitable linker. Specifically, such antibody fragments can be produced by cleaving the antibody with an enzyme (e.g., papain, pepsin) into antibody fragments, or by constructing a gene encoding the antibody fragment and inserting the gene into an expression vector and introducing the resulting recombinant expression vector into a suitable host cell, thereby expressing the antibody fragment (see, for example, Co, M. S., et al., J. Immunol. (1994), 152, 2968-2976; Better, M. & Horwitz, A. H., Methods in Enzymology (1989), 178, 476-496, Academic Press, Inc.; Plueckthun, A. & Skerra, A., Methods in Enzymology (1989) 178, 476-496, Academic Press, Inc.; Lamoyi, E., Methods in Enzymology (1989) 121, 652-663; Rousseaux, J. et al., Methods in Enzymology (1989) 121, 663-669; and Bird, R. E. et al., TIBTECH (1991) 9, 132-137).

A scFv can be produced by linking the H-chain V-region to the L-chain V-region

through a linker, preferably a peptide linker (Huston, J. S. et al., Proc. Natl. Acad. Sci. USA (1988) 85, 5879-5883). The H-chain V-region and the L-chain V-region in the scFv may be derived from any one of the antibodies described herein. The peptide linker which binds the V-regions may be any single chain peptide, for example, of 12-19 amino acid residues.

The DNA encoding the scFv can be prepared by first amplifying a DNA encoding the H-chain V-region and a DNA encoding the L-chain V-region of the antibody separately using a DNA fragment encoding the entire region or a part of the H-chain that includes the V-region and a DNA fragment encoding the entire region or a part of the L-chain that includes the V-region as templates and primer pairs that define the terminal ends of the DNA fragments; and then amplifying a DNA encoding the peptide linker using a DNA fragment encoding the peptide linker as a template and a primer pair that define the terminal ends of the DNA fragment so that each terminal end of the peptide linker is ligated to the H-chain V-region and the L-chain V-region, respectively.

Once the DNA encoding the scFv is prepared, an expression vector carrying the DNA and a host transformed with the expression vector can be prepared by conventional methods. The scFv can be produced from the transformed host by a conventional method.

The fragments of the antibody may be produced by preparing genes for the fragments and expressing the genes in suitable hosts as described above. The antibody fragments is also encompassed in the "antibody" of the present invention.

As a modified form of the above-mentioned antibodies, for example, anti-PTHrP antibody conjugated to any molecule (e.g., polyethylene glycol) may also be used. Such modified antibodies are also encompassed in the "antibody" of the present invention. The modified antibodies can be prepared by chemical modifications of the antibodies. The chemical modification techniques suitable for this purpose have already been established in the art.

6. Expression and production of recombinant antibody or modified antibody

The antibody gene constructed as described above can be produced and expressed by known methods. For the expression in a mammalian cell, a conventional useful promoter, the

antibody gene to be expressed and a poly(A) signal (located downstream to the 3' end of the antibody gene) are operably linked. For example, as the useful promoter/enhancer system, a human cytomegalovirus immediate early promoter/enhancer system may be used.

Other promoter/enhancer systems usable in the expression of the antibody used in the present invention include those derived from viruses (e.g., retrovirus, polyoma virus, adenovirus and simian virus 40 (SV40)) and those derived from mammalian cells (e.g., human elongation factor 1 α (HEF1 α).

When SV40 promoter/enhancer system is used, the gene expression may be performed readily by the method of Mulligan et al. (Nature (1979) 277, 108). When HEF1 α promoter/enhancer system is used, the gene expression may be performed readily by the method of Mizushima et al. (Nucleic Acids Res. (1990) 18, 5322).

For the expression in E. coli, a conventional useful promoter, a signal sequence for secreting the antibody of interest and the antibody gene may be operably linked. As such a promoter, lacZ promoter or araB promoter may be used. When lacZ promoter is used, the gene expression may be performed by the method of Ward et al. (Nature (1098) 341, 544-546; FASBE J. (1992) 6, 2422-2427). When araB promoter is used, the gene expression may be performed by the method of Better et al. (Better et al., Science (1988) 240, 1041-1043).

Regarding the signal sequence for secretion of the antibody, when the antibody of interest is intended to be secreted in a periplasmic space of the E. coli, pelB signal sequence (Lei, S. P. et al., J. Bacteriol. (1987) 169, 4379) may be used. The antibody secreted into the periplasmic space is isolated and then refolded so that the antibody takes an appropriate configuration for use.

Regarding the replication origin, those derived from viruses (e.g., SV40, polyoma virus, adenovirus, bovine papilloma virus (BPV)) or the like may be used. In order to increase the gene copy number in the host cell system, the expression vector may further contain a selective marker gene, such as an aminoglycoside phosphotranferase (APH) gene, a thymidine kinase (TK) gene, an E. coli xanthine-guanine phosphoribosyltransferase (Ecogpt) gene and a dihydrofolate reductase (dhfr) gene.

For the production of the antibody used in the present invention, any expression system

such as eukaryotic and prokaryotic cell systems may be used. The eukaryotic cell includes established cell lines of animals (e.g., mammals, insects, molds and fungi, yeast). The prokaryotic cell includes bacterial cells such as E. coli cells.

It is preferable that the antibody used in the present invention be expressed in a mammalian cell, such as a CHO, COS, myeloma, BHK, Vero or HeLa cell.

Next, the transformed host cell is cultured in vitro or in vivo to produce the antibody of interest. The culturing of the host cell may be performed by any known method. The culture medium usable herein may be DMEM, MEM, RPMI 1640 or IMDM medium. The culture medium may contain a serum supplement, such as fetal calf serum (FCS).

7. Isolation and purification of antibody

The antibody expressed and produced as described above may be isolated from the cells or the host animal body and purified to uniformity. The isolation and purification of the antibody used in the present invention may be performed on an affinity column. Examples of a protein A column include Hyper D, POROS and Sepharose F.F. (Pharmacia). The method is not particularly limited and other methods conventionally used for the isolation and purification of an antibody may also be employed. For example, various chromatographs using columns other than the above-mentioned affinity column, filtration, ultrafiltration, salting out and dialysis may be used singly or in combination to isolate and purify the antibody of interest (Antibodies A Laboratory Manual. Ed. Harlow, David Lane, Cold Spring Harbor Laboratory, 1988).

8. Determination of the activities of the antibody

The determination of the antigen-binding activity (Antibodies A Laboratory Manual, Ed. Harlow, David Lane, Cold Spring Harbor Laboratory, 1988) or the inhibitory activity against a ligand receptor (Harada, A. et al., International Immunology (1993) 5, 681-690) of the antibody used in the present invention may be performed by any known methods.

The method for the determination of the antigen-binding activity of the anti-PTHrP antibody used in the present invention may be ELISA (enzyme-linked immunosorbent assay),

EIA (enzyme immunoassay), RIA (radioimmunoassay) or a fluorescent antibody. For example, when enzyme immunoassay is employed, a sample solution containing the anti-PTHrP antibody (e.g., a culture supernatant of anti-PTHrP antibody-producing cells, or the anti-PTHrP antibody in a purified form) is added to a plate on which PTHrP (1-34) is previously coated. A secondary antibody labeled with an enzyme (e.g., alkaline phosphatase) is further added to the plate. The plate is incubated and washed. A substrate for the enzyme (e.g., p-nitrophenylphosphoric acid) is added to the plate, and the absorbance of the solution in the plate is measured to evaluate the antigen-binding activity of the antibody.

To confirm the activity of the antibody used in the present invention, a neutralizing activity of the antibody (e.g., anti-PTHrP antibody) may be determined.

9. Routes for administration and pharmaceutical preparations

The therapeutic agent of the present invention can be used for the treatment or amelioration of drug-resistant hypercalcemia. The drug-resistant hypercalcemia may be of cancer-induced type or other type. An example of the cancer-induced type is hypercalcemia of malignancy.

Examples of the non-cancer-induced type include perinatal hypercalcemia in gravidas and lactating women and neonatal hypercalcemia.

The therapeutic agent comprising the anti-PTHrP antibody as an active ingredient according to the present invention may be administered orally or parenterally, preferably parenterally. The therapeutic agent may take any dosage form, such as a transpulmonary agent (e.g., an agent administered with the help of a device such as a nebulizer), a nasogastric agent, a transdermic agent (e.g., ointment, cream) or an injection. Examples of the injection include an intervenous injection (e.g., drops), an intramuscular injection, an intraperitoneal injection and a subcutaneous injection for systemic or topical administration. The route of administration may be properly selected depending on the age of a patient and the conditions of diseases. An effective single dose may be selected within the range from 0.001 to 1,000 mg per kg of the body weight, preferably 0.1 to 50 mg per kg of the body weight, more preferably 0.5 to 10 mg per kg of the body weight. Alternatively, the dose to a patient may be selected

within the range from 0.01 to 100,000 mg/body, preferably 1 to 5000 mg/body, more preferably 10 to 500 mg/body. However, the dose of the therapeutic agent comprising the anti-PTHrP antibody of the present invention is not particularly limited to these ranges.

The therapeutic agent may be administered to a patient at any stage, including before or after the development of drug-resistant hypercalcemia. The therapeutic agent may also be administered at the stage where the development of weight loss is predicted in the patient.

The therapeutic agent comprising the anti-PTHrP antibody as an active ingredient of the present invention may be formulated by any conventional method (Remington's Pharmaceutical Science, latest edition, Mark Publishing Company, Easton, USA). The preparation may further comprise pharmaceutically acceptable carriers and additives.

Examples of such carriers and additives include water, pharmaceutically acceptable organic solvents, collagen, polyvinyl alcohol, polyvinyl pyrrolidone, carboxyvinyl polymer, sodium carboxymethyl cellulose, poly(sodium acrylate), sodium arginate, water soluble dextran, sodium carboxymethyl starch, pectin, methyl cellulose, ethyl cellulose, xanthane gum, gum arabic, casein, agar, polyethylene glycol, diglycerin, glycerin, propylene glycol, vaseline, paraffin, stearyl alcohol, stearic acid, human serum albumin (HSA), mannitol, sorbitol, lactose, and surfactants acceptable as pharmaceutical additives.

In the practical use, the additive is properly selected from the above members either singly or in combination depending on (without limitation) the dosage form employed. For example, for use as an injectable form, the anti-PTHrP antibody of the purified form is dissolved in a solvent (e.g., physiological saline, a buffer, a grape sugar solution) and then an adsorption-preventing agent (e.g., Tween 80, Tween 20, a gelatin, human serum albumin) is added thereto. The therapeutic agent of the present invention may also be in a re-constitutable freeze-dried form, which is dissolved before use. For the formulation of the freeze-dried dosage form, an excipient such as a sugar alcohol (e.g., mannitol, grape sugar) or a sugar may be incorporated.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 is a graph showing the results of the pharmacological study in bisphosphonate-resistant hypercalcemia model animals.

Fig. 2 is a graph showing the results of the pharmacological study in bisphosphonate-resistant hypercalcemia model animals.

Fig. 3 is a graph showing the results of the pharmacological study in calcitonin-resistant hypercalcemia model animals.

Fig. 4 is a graph showing the results of the pharmacological study in calcitonin-resistant hypercalcemia model animals.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinbelow, the present invention will be described in greater detail with reference to the following Reference Examples and Examples, which should not be construed as limiting the technical scope of the invention.

[EXAMPLE 1] Pharmacological study in bisphosphonate-resistant hypercalcemia model animals

(1) Objective of the study

To produce a model animal of hypercalcemia resistant to a bisphosphonate using a hypercalcemia model animal (a human tumor-implanted nude rat), and to examine the therapeutic effect of a humanized monoclonal anti-PTHrP antibody on blood calcium level in the model animal.

(2) Methods

As a model animal, a nude rat implanted with human large cell lung carcinoma LC-6 [purchased from the Central Institute for Experimental Animals] was used. It is known that a nude rat implanted with human large cell lung carcinoma LC-6 shows an increased blood calcium level as increasing the tumor volume and develops weight loss and so on. After the development of hypercalcemia was confirmed, the model animal was administered repeatedly with alendronate, which is a kind of bisphosphonate, until no amelioration in blood calcium level was observed, in other ward, until the model animal acquired resistance to the bisphosphonate. In this manner,

bisphosphonate-resistant hypercalcemia model animal was produced. The human large cell lung carcinoma LC-6 was subcultured in vivo using a BALB/c-nu/nu nude mouse (CLEA Japan, Inc.).

The amelioration effect of the humanized monoclonal antibody in the model rats with bisphosphonate-resistant hypercalcemia was evaluated with respect to body weights and blood calcium levels in the rats.

For the evaluation of pharmacological actions of the humanized monoclonal antibody, 5-weeks-old male F344/N Jcl-rnu nude rats (CLEA Japan, Inc.) were purchased and acclimatized for 1 week. The resulting 6-weeks-old rats were implanted with the tumor, and the rats with increased blood calcium levels and reduced body weights were used as hypercalcemia model animals. The bisphosphonate-resistant hypercalcemia model animals were produced and divided into groups in the following manner. The subcultured human large cell lung carcinoma LC-6 was removed from the nude mouse, and then finely cut into 3-mm cube of blocks. The resulting tumor blocks were subcutaneously implanted into each of the rats at the lateral region at a ratio of one piece per mouse. About a month and a half after the implantation, the rats with increased blood calcium levels and reduced body weights (i.e., hypercalcemia model animals) were divided into groups so that blood calcium levels and body weights of the rats in the individual groups were averaged.

Alendronate in the form of a known pharmaceutical preparation for hypercalcemia (alendronate sodium hydrate injection, "Teiroc Injection" from Teijin Limited) was administered to each of a group of the hypercalcemia model animals via the tail vein twice a week at a dose level of 2.5 mg/kg. As a control, phosphate buffered saline (PBS) was administered to each of another group of the hypercalcemia model animals via the tail vein twice a week. In the alendronate-administered model animals, those animals in which no amelioration effect on blood calcium level was observed after five-time administrations of alendronate (i.e., administered on days 0, 3, 7, 10 and 14) were recognized as the bisphosphonate-resistant hypercalcemia model animals. On day 17, the recognized model animals were divided into groups so that blood calcium levels and body weights of the animals in the individual groups were averaged.

Examination of the therapeutic effect on bisphosphonate-resistant hypercalcemia was performed in the following manner. In a test group of the bisphosphonate-resistant hypercalcemia model animals (which were produced and divided into groups as set forth above), either 3 mg/kg of a humanized monoclonal anti-PTHrP antibody or 2.5 mg/kg of alendronate was administered to each model animal via the tail vein. In the control group, PBS was administered to each model animal via the tail vein.

The determination of blood calcium levels and body weights was performed on days 3, 7, 10, 14 and 17 after the administration of either alendronate or PBS. On day 17, the model animals in the alendronate-administered group were divided into groups so that blood calcium levels and body weights of the animals in the individual groups were averaged. In each of the test groups, either a humanized monoclonal antibody or alendronate was administered. In the control group, PBS was administered. On days 4 and 7 after the administration of the humanized monoclonal antibody or alendronate, the blood calcium level and the body weight were measured for each animal to determine the pharmacological efficacy.

The blood calcium level was determined as a whole blood ionized calcium level, by drawing blood from each of the animals via the tail vein using a hematocrit tube and applying the blood to 643 model Automatic Ca/pH Analyzer (CIBA-CORNING).

(3) Results

Model animals of bisphosphonate-resistant hypercalcemia were successfully produced by repeatedly administrating a bisphosphonate five times in total at intervals of twice a week. The humanized monoclonal antibody of the present invention could ameliorate the blood calcium levels in the bisphosphonate-resistant hypercalcemia model animals (Fig. 1). In the antibody-administered group, the restoration of body weight was also observed (Fig. 2). From these results, it is found that the humanized monoclonal anti-PTHrP antibody is useful as a therapeutic agent for hypercalcemia of malignancy that acquires resistance to a bisphosphonate.

[EXAMPLE 2] Pharmacological test in calcitonin-resistant hypercalcemia model animals

(1) Objective of the test

To produce a model animal of hypercalcemia resistant to a calcitonin using a hypercalcemia model animal (a human tumor-implanted nude rat), and to examine the therapeutic effect of a humanized monoclonal anti-PTHrP antibody on blood calcium level in the model animal.

(2) Methods

As a model animal, a nude rat implanted with human large cell lung carcinoma LC-6 [purchased from the Central Institute for Experimental Animals] was used. It is known that a nude rat implanted with human large cell lung carcinoma LC-6 shows an increased blood calcium level as increasing the tumor volume and develops weight loss and so on. After the development of hypercalcemia was confirmed, the model animal was repeatedly administered with elcatonin, which is a kind of calcitonin, until no amelioration in blood calcium level was observed, in other ward, until the model animal acquired resistance to the calcitonin. In this manner, calcitonin-resistant hypercalcemia model animal was produced. The human large cell lung carcinoma LC-6 was subcultured in vivo using a BALB/c-nu/nu nude mouse (CLEA Japan, Inc.).

The amelioration effect of the humanized monoclonal antibody in the model rats with calcitonin-resistant hypercalcemia was evaluated with respect to body weights and blood calcium levels in the rats.

For the evaluation of pharmacological actions of the humanized monoclonal antibody, 5-weeks-old male F344/N Jcl-rnu nude rats (CLEA Japan, Inc.) were purchased and acclimatized for 1 week. The resulting 6-weeks-old rats were implanted with the tumor, and the rats with increased blood calcium levels and reduced body weights were used as hypercalcemia model animals. The calcitonin-resistant hypercalcemia model animals were produced and divided into groups in the following manner. The subcultured human large cell lung carcinoma LC-6 was removed from the nude mouse, and then finely cut into 3-mm cube of blocks. The resulting tumor blocks were subcutaneously implanted into each of the rats at the lateral region at a ratio of one piece per mouse. About a month and a half after the

implantation, the rats with increased blood calcium levels and reduced body weights (i.e., hypercalcemia model animals) were divided into groups so that blood calcium levels and body weights of the rats in the individual groups were averaged.

Elcatonin ("Elcitonin Injection" from Asahi Chemical Industry Co., Ltd.) was administered to each of a group of the hypercalcemia model animals via the tail vein twice a day at a dose level of 10 U/kg (at intervals of 12 hours). As a control, PBS was administered to each of another group of the hypercalcemia model animals via the tail vein twice a week. In the elcatonin-administered model animals, those animals in which no amelioration effect on blood calcium level was observed after twelve-time administrations of elcatonin (i.e., administered on days 0, 1, 2, 3, 4 and 5 twice a day) were recognized as the elcatonin-resistant hypercalcemia model animals. The recognized model animals were divided into groups so that blood calcium levels and body weights of the animals in the individual groups were averaged.

Examination of the therapeutic effect on elcitonin-resistant hypercalcemia was performed in the following manner. In a test group of the elcitonin-resistant hypercalcemia model animals (which were produced and divided into groups as set forth above), either a humanized monoclonal anti-PTHrP antibody (3 mg/kg) or PBS was administered to each model animal via the tail vein. In the control group, PBS was administered to each model animal via the tail vein.

The determination of blood calcium levels was performed on days 0.5, 1, 2, 3, 4, 5 and 6 after the administration of either elcatonin or PBS. The determination of body weights was performed on days 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 after the administration of either elcatonin or PBS. On day 6, the model animals in the elcatonin-administered group were divided into groups so that blood calcium levels and body weights of the animals in the individual groups were averaged. In each of test groups, either a humanized monoclonal antibody or PBS was administered. In the control group, PBS was administered. On days 1 and 3 after the administration of the humanized monoclonal antibody or PBS, the blood calcium level and the body weight were measured for each animal to determine the pharmacological efficacy. The blood calcium level was determined as a whole blood ionized

calcium level, by drawing blood from each of the animals via the tail vein using a hematocrit tube and applying the blood to 643 model Automatic Ca/pH Analyzer (CIBA-CORNING).

(3) Results

Model animals for calcitonin-resistant hypercalcemia were successfully produced by successively administrating a calcitonin twelve times in total at intervals of twice a day. The humanized monoclonal antibody successfully ameliorated the blood calcium levels in the calcitonin-resistant hypercalcemia model animals (Fig. 3). In the antibody-administered group, the restoration of body weight was also observed (Fig. 4). From these results, it is found that the humanized monoclonal anti-PTHrP antibody is useful as a therapeutic agent for hypercalcemia of malignancy which acquires resistance to a calcitonin.

[REFERENCE EXAMPLE 1]

Preparation of hybridomas producing anti-PTHrP (1-34) mouse monoclonal antibody

Hybridomas capable of producing a monoclonal antibody against human PTHrP (1-34) (SEQ ID NO: 75), #23-57-154 and #23-57-137-1, were prepared as follows (see Sato, K. et al., J. Bone Miner. Res. 8, 849-860, 1993). The amino acid sequence of the human PTHrP (1-34) is shown in SEQ ID NO:75.

For use as an immunogen, PTHrP (1-34) (Peninsula) was conjugated with a carrier protein thyroglobulin using carbodiimide (Dojinn). The thycloglobulin-conjugated PTHrP (1-34) was dialyzed to obtain a solution having a protein concentration of 2 μg/ml. The resulting solution was mixed with Freund's adjuvant (Difco) at a mixing ratio of 1:1 to give an emulsion. This emulsion was injected to 16 female BALB/C mice 11 times subcutaneously at the back or intraperitoneally at a dose level of 100 μg/mouse for each injection, thereby immunizing the mice. For the priming immunization, Freund's complete adjuvant was used; while for the boosting immunization, Freund's incomplete adjuvant was used.

Each of the immunized mice was determined for its antibody titer in the serum in the following manner. That is, each of the mice was blood-drawn via its tail vein, and the anti-

serum is separated from the blood. The anti-serum was diluted with a RIA buffer and mixed with ^{125}I-labeled PTHrP (1-34) to determine the binding activity. The mice that were confirmed to have a sufficiently increased titer were injected with PTHrP (1-34) without a carrier protein intraperitoneally at a dose level of 50 μ g/mouse for the final immunization.

Three days after the final immunization, the mouse is sacrificed and the spleen was removed therefrom. The spleen cells were subjected to cell fusion with mouse myeloma cell line P3x63Ag8U.1 in accordance with a conventional known method using 50% polyethylene glycol 4000. The fused cells thus prepared were seeded to each well of eighty-five 96-well plates at a density of 2 x 10^4/well. Hybridomas were screened in HAT medium as follows.

The screening of hybridomas was performed by determining the presence of PTHrP-recognition antibodies in the culture supernatant of the wells in which cell growth had been observed in HAT medium, by solid phase RIA method. The hybridomas were collected from the wells in which the binding ability to the PTHrP-recognition antibodies had been confirmed. The hybridomas thus obtained was suspended into RPMI-1640 medium containing 15% FCS supplemented with OPI-supplement (Sigma), followed by unification of the hybridomas by limiting dilution method. Thus, two types of hybridoma clones, #23-57-154 and #23-57-137-1, could be obtained, both which had a high binding ability to PTHrP (1-34).

Hybridoma clone #23-57-137-1 was designated "mouse-mouse hybridoma #23-57-137-1", and has been deposited under the terms of the Budapest Treaty on August 15, 1996 at the National Institute of Bioscience and Human-technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) under the accession No. FERM BP-5631.

[REFERENCE EXAMPLE 2]

Cloning of DNAs encoding V-regions of mouse monoclonal antibody against human PTHrP (1-34)

Cloning of DNAs encoding the V-regions of a mouse monoclonal antibody against human PTHrP (1-34), #23-57-137-1, was performed in the following manner.

(1) Preparation of mRNA

mRNA from hybridoma #23-57-137-1 was prepared using Quick Prep mRNA Purification Kit (Pharmacia Biotech). That is, cells of hybridoma #23-57-137-1 were fully homogenized with an extraction buffer, and mRNA was isolated and purified therefrom on an oligo(dT)-Cellulose Spun Column in accordance with the instructions included in the kit. The resulting solution was subjected to ethanol precipitation to obtain the mRNA as a precipitate. The mRNA precipitate was dissolved in an elution buffer.

(2) Production and amplification of cDNA for gene encoding mouse H-chain V-region

(i) Cloning of cDNA for #23-57-137-1 antibody H-chain V-region

A gene encoding H-chain V-region of the mouse monoclonal antibody against human PTHrP was cloned by 5'-RACE method (Frohman, M. A. et al., Proc. Natl. Acad. Sci. USA, 85, 8998-9002, 1988; Belyavsky, A. et al., Nucleic Acids Res. 17, 2919-2932, 1989). The 5'-RACE method was performed using 5'-Ampli FINDER RACE Kit (CLONETECH) in accordance with the instructions included in the kit. In this method, the primer used for synthesis of cDNA was MHC2 primer (SEQ ID NO: 1) which is capable of hybridizing to mouse H-chain C-region. The above-prepared mRNA (about 2 μg), which was a template for the cDNA synthesis, was mixed with MHC2 primer (10 pmoles). The resulting mixture was reacted with a reverse transcriptase at 52° C for 30 minuets to effect the reverse transcription of the mRNA into cDNA.

The resulting reaction solution was added with 6N NaOH to hydrolyze any RNA remaining therein (at 65° C for 30 min.) and then subjected to ethanol precipitation to isolate and purify the cDNA as a precipitate. The purified cDNA was ligated to Ampli FINDER Anchor (SEQ ID NO: 42) at the 5' end by reacting with T4 RNA ligase at 37° C for 6 hours and additionally at room temperature for 16 hours. As the primers for amplification of the cDNA by PCR method, Anchor primer (SEQ ID NO: 2) and MHC-G1 primer (SEQ ID NO: 3) (S.T. Jones, et al., Biotechnology, 9, 88, 1991) were used.

The PCR solution comprised (per 50 μl) 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25

mM dNTPs (dATP, dGTP, dCTP, dTTP), 1.5 mM $MgCl_2$, 2.5 units of TaKaRa Taq (Takara Shuzo Co., Ltd.), 10 pmoles Anchor primer, and 1 μl of the reaction mixture of the cDNA to which MHC-G1 primer and Ampli FINDER Anchor primer had been ligated, over which mineral oil (50 μl) was layered. The PCR was performed on Thermal Cycler Model 480J (Perkin Elmer) for 30 cycles under the conditions: 94° C for 45 sec.; 60° C for 45 sec.; and 72° C for 2 min.

(ii) Cloning of cDNA for #23-57-137-1 antibody L-chain V-region

A gene encoding L-chain V-region of the mouse monoclonal antibody against human PTHrP was cloned by 5'-RACE method (Frohman, M. A. et al., Proc. Natl. Acad. Sci. USA, 85, 8998-9002, 1988; Belyavsky, A. et al., Nucleic Acids Res. 17, 2919-2932, 1989). The 5'-RACE method was performed using 5'-Ampli Finder RACE Kit (CLONETECH) in accordance with the instructions included in the kit. In this method, oligo-dT primer was used as the primer for synthesizing cDNA. The above-prepared mRNA (about 2 μg), which was a template for the cDNA synthesis, was mixed with oligo-dT primer. The resulting mixture was reacted with a reverse transcriptase at 52° C for 30 min. to effect the reverse transcription of the mRNA into cDNA. The resulting reaction solution was added with 6N NaOH to hydrolyze any RNA remaining therein (at 65° C for 30 min.). The resulting solution was subjected to ethanol precipitation to isolate and purified the cDNA as a precipitate. The cDNA thus synthesized was ligated to Ampli FINDER Anchor at the 5' end by reacting with T4 RNA ligase at 37° C for 6 hours and additionally at room temperature for 16 hours.

A PCR primer MLC (SEQ ID NO: 4) was designed based on the conserved sequence of mouse L-chain λ chain C-region and then synthesized using 394 DNA/RNA Synthesizer (ABI). The PCR solution comprised (per 100 μl) 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.25 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1.5 mM $MgCl_2$, 2.5 units of AmpliTaq (PERKIN ELMER), 50 pmoles of Anchor primer (SEQ ID NO: 2), and 1 μl of the reaction mixture of the cDNA to which MLC (SEQ ID NO: 4) and Ampli FINDER Anchor were ligated, over which mineral oil (50 μl) was layered. The PCR reaction was performed on Thermal Cycler Model 480J (Perkin Elmer) for 35 cycles under the conditions: 94° C for 45 sec.; 60° C for

45 sec.; and 72° C for 2 min.

(3) Purification and fragmentation of PCR products

Each of the DNA fragments amplified by PCR method described above was separated by agarose gel electrophoresis on a 3% Nu Sieve GTG agarose (FMC Bio. Products). For each of the H-chain V-region and the L-chain V-region, an agarose gel segment containing a DNA fragment of about 550 bp was excised from the gel. Each of the gel segments was subjected to purification of the DNA fragment of interest using GENECLEAN II Kit (BIO101) in accordance with the instructions included in the kit. The purified DNA was precipitated with ethanol, and the DNA precipitate was dissolved in 20 μl of a solution containing 10 mM Tris-HCl(pH 7.4) and 1 mM EDTA. An aliquot (1 μl) of the DNA solution was digested with a restriction enzyme XmaI (New England Biolabs) at 37° C for 1 hour and further digested with a restriction enzyme EcoRI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The digestion solution was extracted with phenol and chloroform and then precipitated with ethanol to collect the DNA.

In this manner, two DNA fragments containing a gene encoding mouse H-chain V-region and a gene encoding mouse L-chain V-region, respectively, were obtained, both which had an EcoRI recognition sequence on the 5' end and an XmaI recognition sequence on the 3' end.

The EcoRI-XmaI DNA fragments containing a gene encoding mouse H-chain V-region and a gene encoding mouse L-chain V-region, respectively, were separately ligeted to pUC19 vector that had been digested with EcoRI and XmaI at 16° C for 1 hour using DNA Ligation Kit ver.2 (Takara Shuzo Co., Ltd.) in accordance with the instructions included in the kit. An aliquot (10 μl) of the ligation mixture was added to 100 μl of a solution containing competent cells of E. coli, JM 109 (Nippon Gene Co., Ltd.). The cell mixture was allowed to stand on ice for 15 min., at 42° C for 1 min. and additionally for 1 min. on ice. The resulting cell mixture was added with 300 μl of SOC medium (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) and then incubated at 37° C for 30 min. The resulting cell solution was plated on LB agar medium or 2xYT agar

medium (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) containing either 100 or 50 μg/ml of ampicillin, 0.1 mM of IPTG and 20 μg/ml of X-gal, and then incubated at 37° C overnight. In this manner, E. coli transformants were prepared.

The transformants were cultured at 37° C overnight in 2 ml of LB or 2xYT medium containing either 100 or 50 μg/ml of ampicillin. The cell fraction was applied to Plasmid Extracter PI-100((Kurabo Industries, Ltd.) or QIAprep Spin Plasmid Kit (QIAGEN) to give a plasmid DNA. The plasmid DNA was sequenced as follows.

(4) Sequencing of genes encoding mouse antibody V-regions

The nucleotide sequence of the cDNA coding region carried on the plasmid was determined in DNA Sequencer 373A (ABI; Perkin-Elmer) using Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). M13 Primer M4 (Takara Shuzo Co., Ltd.) (SEQ ID NO: 5) and M13 Primer RV (Takara Shuzo Co., Ltd.) (SEQ ID NO: 6) were used as the primers for sequencing, and the nucleotide sequence was confirmed in the both directions.

The plasmid containing a gene encoding mouse H-chain V-region derived from hybridoma #23-57-137-1 was designated "MBC1H04", and the plasmid containing a gene encoding mouse L-chain V-region derived from hybridoma #23-57-137-1 was designated "MBC1L24". The nucleotide sequences (including the corresponding amino acids sequences) of the gene encoding the mouse #23-57-137-1 antibody-derived H-chain V-region in plasmid MBC1H04 and the gene encoding the mouse #23-57-137-1 antibody-derived L-chain V-region in plasmid MBC1H24 were shown in SEQ. ID Nos: 57 and 65, respectively. The amino acid sequences of the polypeptides for the H-chain V-region and the L-chain V-region were shown in SEQ. ID NOs: 46 and 45, respectively.

The E. coli strain containing plasmid MBC1H04 and the E. coli strain containing plasmid MBC1L24 were designated "Escherichia coli JM109 (MBC1H04)" and "Escherichia coli JM109 (MBC1L24)", respectively. These E. coli strains have been deposited under the terms of the Budapest Treaty at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan (1-3, Higashi 1-chome, Tsukuba-shi,

Ibaraki, Japan) on August 15, 1996, under the Accession No. FERM BP-5628 for Escherichia coli JM109 (MBC1H04) and FERM BP-5627 for Escherichia coli JM109 (MBC1L24), respectively.

(5) Determination of CDRs of mouse monoclonal antibody #23-57-137-1 against human PTHrP

The H-chain V-region and the L-chain V-region have general structures similar to each other, each of which has four framework regions (FRs) linked through three hypervariable regions (i.e., complementarity determining regions; CDRs). The amino acid sequences of the FRs are relatively well conserved, while the amino acid sequence of the CDRs have an extremely high variability (Kabat, E.A. et al., "Sequence of Proteins of Immunological Interest", US Dept. Health and Human Services, 1983).

In view of these facts, the homology in amino acid between the V-regions of the mouse monoclonal antibody against human PTHrP was determined with reference to the database of amino acid sequences of antibodies established by Kabat et al. Thus, the CDRs of the V-regions were determined as shown in Table 1.

The amino acid sequences of CDRs 1-3 in the L-chain V-region are shown in SEQ ID Nos: 59 to 61, respectively; and the amino acid sequences of CDRs 1-3 in the H-chain V-region are shown in SEQ ID Nos: 62 to 64, respectively.

Table 1

V-region	SEQ ID NO.	CDR1	CDR2	CDR3
H-chain V-region	57	31-35	50-66	99-107
L-chain V-region	65	23-34	50-60	93-105

[REFERENCE EXAMPLE 3] Construction of Chimeric Antibody

(1) Construction of chimeric antibody H-chain

(i) Construction of H-chain V-region

To ligate to an expression vector carrying a genomic DNA of human H-chain C-region C γ 1, the cloned DNA encoding mouse H-chain V-region was modified by PCR method. A backward primer MBC1-S1 (SEQ ID NO: 7) was designed to hybridize to a DNA sequence encoding the 5' region of the leader sequence of the V-region and to have both a Kozak consensus sequence (Kozak, M. et al., J. Mol. Biol., 196, 947-950, 1987) and a HindIII-recognition sequence. A forward primer MBC1-a (SEQ ID NO: 8) was designed to hybridize to a DNA sequence encoding the 3' region of the J region and to have both a donor splice sequence and a BamHI-recognition sequence. The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and a buffer appended thereto. The PCR solution comprised (per 50 μ l) 0.07 μ g of plasmid MBC1H04 as a template DNA, 50 pmoles of MBC1-a and 50 pmoles of MBC1-S1 as primers, 2.5U of TaKaRa Ex Taq and 0.25 mM dNTPs in the buffer, over which 50 μ l of mineral oil was layered. The PCR was run for 30 cycles under the conditions: 94° C for 1 min.; 55° C for 1 min.; 72° C for 2 min. The DNA fragments thus amplified by the PCR method were separated by agarose gel electrophoresis on a 3% Nu Sieve GTG Agarose (FMC Bio. Products).

Then, an agarose gel segment containing a DNA fragment of 437 bp was excised, and the DNA fragment was purified therefrom using GENECLEAN II Kit (BIO101) in accordance with the instructions included in the kit. The purified DNA was collected by ethanol precipitation, and then dissolved in 20 μ l of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. An aliquot (1 μ l) of the resulting DNA solution was digested with restriction enzymes BamHI and HindIII (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The digestion solution was extracted with phenol and chloroform and then precipitated with ethanol to collect the DNA of interest.

The obtained HindIII-BamHI DNA fragment, which containing a gene encoding the mouse H-chain V-region, was subcloned into pUC19 vector that had been digested with HindIII and BamHI. The resulting plasmid was sequenced on DNA Sequencer 373A (Perkin-Elmer) using M13 Primer M4 and M13 Primer RV as primers and Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). As a result, a plasmid which carried a gene of correct

nucleotide sequence encoding the mouse H-chain V-region derived from hybridoma #23-57-137-1 and had a HindIII-recognition sequence and a Kozak sequence on its 5' region and a BamHI-recognition sequence on its 3' region was obtained, which was designated "MBC1H/pUC19".

(ii) Construction of H-chain V-region for preparation of cDNA-type of mouse-human chimeric H-chain

To ligate to cDNA of the human H-chain C-region C γ 1, the DNA encoding the mouse H-chain V-region constructed as described above was modified by PCR method. A backward primer MBC1HVS2 (SEQ ID NO: 9) for the V-region was designed to cause the replacement of the second amino acid (asparagine) of the sequence encoding the front part of the leader sequence of the H-chain V-region by glycine and to have a Kozak consensus sequence (Kozak, M. et al., J. Mol. Biol., 196, 947-950, 1987) and HindIII- and EcoRI-recognition sequences. A forward primer MBC1HVR2 (SEQ ID NO: 10) for the H-chain V-region was designed to hybridize to a DNA sequence encoding the 3' region of the J region, to encoding the 5' region of the C-region and to have ApaI- and SmaI-recognition sequences.

The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and a buffer appended thereto. The PCR solution comprised (per 50 μ l) 0.6 μ g of plasmid MBC1H/pUC19 as a template DNA, 50 pmoles of MBC1HVS2 and 50 pmoles of MBC1HVR2 as primers, 2.5U of TaKaRa Ex Taq and 0.25 mM of dNTPs in the buffer, over which 50 μ l of mineral oil was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min.; 55° C for 1 min.; 72° C for 1 min. The DNA fragments amplified by the PCR reaction were separated by agarose gel electrophoresis on a 1% Sea Kem GTG Agarose (FMC Bio. Products). Then, an agarose gel segment containing a DNA fragment of 456 bp was excised and the DNA fragment was purified therefrom using GENECLEAN II Kit (BIO101) in accordance with the instructions included in the kit. The purified DNA was precipitated with ethanol and then dissolved in 20 μ l of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The resulting DNA solution (1 μ g) was digested with restriction enzymes EcoRI and

SmaI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The digestion solution was extracted with phenol and chloroform and then precipitated with ethanol to collect the DNA. The obtained EcoRI-SmaI DNA fragment, which containing a gene encoding the mouse H-chain V-region, was subcloned into pUC19 vector that had been digested with EcoRI and SmaI. The resulting plasmid was sequenced on DNA Sequencer 373A (Perkin-Elmer) using M13 Primer M4 and M13 Primer RV, and Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). As a result, a plasmid which contained a gene of correct nucleotide sequence encoding mouse H-chain V-region derived from hybridoma #23-57-137-1 and had EcoRI- and HindIII-recognition sequences and a Kozak sequence on its 5' region and ApaI- and SmaI-recognition sequences on its 3' region was obtained, which was designated "MBC1Hv/pUC19".

(iii) Construction of expression vector for chimeric antibody H-chain

cDNA containing the DNA for human antibody H-chain C-region C γ 1 was prepared as follows. mRNA was prepared from a CHO cell into which both an expression vector DHFR-ΔE-RVh-PM-1-f (see WO 92/19759) encoding the genomic DNAs of humanized PM1 antibody H-chain V-region and human antibody H-chain C-region IgG1 (N. Takahashi et al., Cell 29, 671-679, 1982) and an expression vector RV1-PM1a (see WO 92/19759) encoding the genomic DNAs of humanized PM1 antibody L-chain V-region and human antibody L-chain κ chain C-region had been introduced. Using the mRNA, cDNA containing the humanized PM1 antibody H-chain V-region and the human antibody C-region C γ 1 was cloned by RT-PCR method, and then subcloned into plasmid pUC19 at the HindIII-BamHI site. After sequencing, a plasmid which had the correct nucleotide sequence was obtained, which was designated "pRVh-PM1f-cDNA".

An expression vector DHFR-ΔE-RVh-PM-1-f in which both a HindIII site located between SV40 promoter and a DHFR gene and an EcoRI site located between EF-1 α promoter and a humanized PM1 antibody H-chain V-region gene had been deleted, was prepared for the construction of an expression vector for cDNA containing the humanized PM1 antibody H-chain V-region gene and the human antibody C-region C γ 1 gene.

The plasmid obtained (pRVh-PM1f-cDNA) was digested with BamHI, blunt-ended

with Klenow fragment, and further digested with HindIII, thereby obtaining a blunt-ended HindIII-BamHI fragment. The blunt-ended HindIII-BamHI fragment was ligated to the above-mentioned HindIII site- and EcoRI site-deleted expression vector DHFR-ΔE-RVh-PM1-f that had been digested with HindIII and BamHI. Thus, an expression vector RVh-PM1f-cDNA was constructed which contained cDNA encoding the humanized PM1 antibody H-chain V-region and the human antibody C-region C γ 1.

The expression vector RVh-PM1f-cDNA containing the cDNA encoding the humanized PM1 antibody H-chain V-region and the human antibody C-region C γ 1 was digested with ApaI and BamHI, and a DNA fragment containing the H-chain C-region was collected therefrom. The resulting DNA fragment was introduced into the plasmid MBC1Hv/pUC19 that had been digested with ApaI and BamHI. The plasmid thus prepared was designated "MBC1HcDNA/pUC19". This plasmid contained cDNA encoding the mouse antibody H-chain V-region and the human antibody C-region C γ 1, and had EcoRI- and HindIII-recognition sequences on its 5' region and a BamHI-recognition sequence on its 3' region.

The plasmid MBC1HcDNA/pUC19 was digested with EcoRI and BamHI to give a DNA fragment comprising a nucleotide sequence encoding the chimeric antibody H-chain. The resulting DNA fragment was introduced into an expression vector pCOS1 that had been digested with EcoRI and BamHI, thereby giving an expression vector for the chimeric antibody, which was designated "MBC1HcDNA/pCOS1". Here, the expression vector pCOS1 was constructed using HEF-PMh-g γ 1 (see WO 92/19759) by deleting therefrom an antibody genes by digestion with EcoRI and SmaI, and then ligating it to EcoRI-NotI-BamHI Adaptor (Takara Shuzo Co., Ltd.).

For preparing a plasmid for the expression in a CHO cell, the plasmid MBC1HcDNA/pUC19 was digested with EcoRI and BamHI to obtain a DNA fragment containing a gene for the chimeric antibody H-chain. The DNA fragment was then introduced into an expression plasmid pCHO1 that had been digested with EcoRI and BamHI to give an expression plasmid for the chimeric antibody, which was designated "MBC1HcDNA/pCHO1". Here, the expression vector pCHO1 was constructed using DHFR-

ΔE-rvH-PM1-f (see WO 92/19759) by deleting therefrom an antibody gene by digestion with EcoRI and SmaI, and then ligating it to EcoRI-NotI-BamHI Adaptor (Takara Shuzo Co., Ltd.).

(2) Construction of human L-chain C-region

(i) Preparation of cloning vector

To construct pUC19 vector containing a gene for human L-chain C-region, a HindIII site-deleted pUC19 vector was prepared. pUC19 vector (2 μg) was digested in 20 μl of a reaction solution containing 20 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, 8 U of HindIII (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The resulting digestion solution was extracted with phenol and chloroform, and then subjected to ethanol precipitation to collect the DNA of interest.

The DNA collected was reacted in 50 μl of a reaction solution containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, 0.5 mM dNTPs and 6U of Klenow fragment (GIBCO BRL) at room temperature for 20 min., thereby rendering the terminal ends of the DNA blunt. This reaction mixture was extracted with phenol and chloroform and then subjected to ethanol precipitation to collect the vector DNA.

The vector DNA thus collected was reacted in 10 μl of a reaction solution containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (v/v) polyethylene glycol-8000 and 0.5 U of T4 DNA ligase (GIBCO BRL) at 16° C for 2 hours, to cause self-ligation of the vector DNA. The reaction solution (5 μl) was added to 100 μl of a solution containing competent cells of E. coli, JM109 (Nippon Gene Co., Ltd.), and the resulting solution was allowed to stand on ice for 30 min., at 42° C for 1 min., and additionally on ice for 1 min. SOC culture medium (500 μl) was added to the reaction solution and then incubated at 37° C for 1 hour. The resulting solution was plated on 2xYT agar medium (containing 50 μg/ml of ampicillin) on which X-gal and IPTG had been applied (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989), and then cultured at 37° C overnight, thereby obtaining a transformant.

The transformant was cultured in 2xYT medium (20 ml) containing ampicillin (50 μg/ml) at 37° C overnight. From the cell fraction of the culture medium, a plasmid DNA

was isolated and purified using Plasmid Mini Kit (QIAGEN) in accordance with the instructions included in the kit. The purified plasmid was digested with HindIII. The plasmid that was confirmed to have a HindIII site-deletion was designated "pUC19 ΔHindIII".

(ii) Construction of DNA encoding human L-chain λ chain C-region

Human antibody L-chain λ chain C-region is known to have at least four isotypes including $Mcg^+Ke^+Oz^-$, $Mcg^-Ke^-Oz^-$, $Mcg^-Ke^-Oz^+$ and $Mcg^-Ke^+Oz^-$ (P. Dariavach, et al., Proc. Natl. Acad. Sci. USA, 84, 9074-9078, 1987). A search was made for a human antibody L-chain λ chain C-region homologous to the #23-57-137-1 mouse L-chain λ chain C-region from the EMBL database. As a result, it was found that the isotype $Mcg^+Ke^+Oz^-$ of the human antibody L-chain λ chain (Accession No. X57819) (P. Dariavach, et al., Proc. Natl. Acad. Sci. USA, 84, 9074-9078, 1987) showed the highest degree of homology to the #23-57-137-1 mouse L-chain λ chain C-region, with a 64.4% homology in terms of amino acid sequence and a 73.4% homology in terms of nucleotide sequence.

Then, a gene encoding the human antibody L-chain λ chain C-region was constructed by PCR method. The primers for the PCR were synthesized using 394 DNA/RNA Synthesizer (ABI). The synthesized primers were as follows: HLAMB1 (SEQ ID NO: 11) and HLAMB3 (SEQ ID NO: 13), both having a sense DNA sequence; and HLAMB2 (SEQ ID NO: 12) and HLAMB4 (SEQ ID NO: 14), both having an antisense DNA sequence; each primer containing a complementary sequence of 20-23 bp on the both terminal ends.

External primers HLAMBS (SEQ ID NO: 15) and HLAMBR (SEQ ID NO: 16) had sequences homologous to the primers HLAMB1 and HLAMB4, respectively. HLAMBS contained EcoRI-, HindIII- and BlnI-recognition sequences, and HLAMBR contained an EcoRI-recognition sequence. In the first-round PCR reaction, the reactions between HLAMB1 and HLAMB2 and between HLAMB3 and HLAMB4 were performed. After the reactions were completed, both of the resulting PCR products were mixed in equivalent quantities, and then assembled in the second-round PCR reaction. The reaction solution was added with the external primers HLAMBS and HLAMBR. This reaction mixture was subjected to the third-round PCR reaction to amplify the full length DNA.

Each PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) in accordance with the instructions included in the kit. In the first-round PCR reaction, 100 μl of either a reaction solution containing 5 pmoles of HLAMB1, 0.5 pmole of HLAMB2 and 5U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) or a reaction solution containing 0.5 pmole of HLAMB3, 5 pmoles of HLAMB4 and 5U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) was used, over which 50 μl of mineral oil was layered. The PCR reaction was run for 5 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min.

In the second-round PCR reaction, a mixture of both the reaction solutions (50 μl each) was used, over which 50 μl of mineral oil was layered. The PCR reaction was run for 3 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min.

In the third-round PCR reaction, the reaction solution to which the external primers HLAMBS and HLAMBR (50 pmoles each) were added was used. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min.

The DNA fragment obtained by the third-round PCR reaction was subjected to electrophoresis on a 3% low-melting agarose gel (NuSieve GTG Agarose, FMC), and separated and purified from the gel using GENECLEAN II Kit (BIO101) in accordance with the instructions included in the kit.

The DNA fragment obtained was digested in a reaction solution (20 μl) containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl and 8U of EcoRI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The digestion solution was extracted with phenol and chloroform, and the DNA was collected therefrom by the ethanol precipitation. The DNA was dissolved in a solution (8 μl) containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The above-prepared plasmid pUC19 ΔHindIII (0.8 μg) was digested with EcoRI in the same manner as set forth above. The digestion solution was subjected to phenol/chloroform extraction and then ethanol precipitation, thereby giving a digested plasmid pUC19 ΔHindIII. The digested plasmid was reacted in a reaction solution (50 μl) containing 50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$ and alkaline phosphatase (E. coli C75; Takara Shuzo Co., Ltd.) at 37° C for 30 min. to dephosphorylate (i.e., BAP-treat) the plasmid.

The reaction solution was subjected to phenol/chloroform extraction, and the DNA was collected therefrom by ethanol precipitation. The DNA thus obtained was dissolved in a solution (10 μl) containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The BAP-treated plasmid pUC19 ΔHindIII (1 μl) was ligated to the above-obtained PCR product (4 μl) using DNA Ligation Kit Ver.2 (Takara Shuzo Co., Ltd.). The resulting plasmid was introduced into a competent cell of E. coli, JM109, to give a transformant. The transformant was cultured overnight in 2xYT medium (2 ml) containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was isolated using QIAprep Spin Plasmid Kit (QIAGEN).

The plasmid obtained was sequenced for the cloned DNA part. The sequencing was performed on 373A DNA Sequencer (ABI) using M13 Primer M4 and M13 Primer RV (Takara Shuzo Co., Ltd.). As a result, it was found that the cloned DNA had a 12-bp deletion therein. The plasmid was designated "C λ Δ/pUC19". Then, for making up for the deleted part, primers HCLMS (SEQ ID NO: 17) and HCLMR (SEQ ID NO: 18) were newly synthesized, and a DNA of correct sequence was reconstructed using these primers by PCR method.

In the first-round PCR reaction, the plasmid C λ Δ/pUC19 having the DNA deletion therein was used as a template, and the reaction was performed with each of the primer sets of HLAMBS and HCLMS and HCLMS and HLAMB4. The PCR products were purified separately. In the second-round PCR reaction, the PCR products were assembled together. In the third-round PCR reaction, the reaction product of the second-round PCR reaction was added with external primers HLAMBS and HLAMB4 and amplified to give the full length DNA.

In the first-round PCR reaction, a reaction solution (100 μl) containing 0.1 μg of C λ Δ/pUC19 as a template, either 50 pmoles of each of the primers HLAMBS and HCLMR or 50 pmoles of each of the primers HCLMS and HLAMB4, and 5U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) was used, over which 50 μl of mineral oil was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72 ° C for 1 min.

The PCR products of the first-round PCR reaction, HLAMBS-HCLMR (236 bp) and

HCLMS-HLAMB4 (147 bp), were subjected to electrophoresis separately on a 3% low-melting agarose gel to isolate the DNA fragments. The DNA fragments were collected and purified from the gels using GENECLEAN II Kit (BIO101). In the second-round PCR reaction, 20 μl of a reaction solution containing 40 ng of each of the purified DNA fragments and 1U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) was used, over which 25 μl of mineral oil was layered. The PCR reaction was run for 5 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min.

In the third-round PCR reaction, 100 μl of a reaction solution containing 2 μl of the reaction solution obtained by the second-round PCR reaction, 50 pmoles of each of external primers HLAMBS and HLAMB4 and 5U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) was used, over which 50 μl of mineral oil was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min., 60° C for 1 min. and 72° C for 1 min., thereby obtaining a DNA fragment of 357 bp (the third PCR product). The DNA fragment was subjected to electrophoresis on a 3% low-melting agarose gel to isolate the DNA fragment. The resulting DNA fragment was collected and purified using GENECLEAN Kit (BIO101).

An aliquot (0.1 μg) of the DNA fragment thus obtained was digested with EcoRI, and then subcloned into plasmid pUC19 ΔHindIII that had been BAP-treated. The resulting plasmid was introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant was cultured overnight in 2 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was isolated and purified using QIAprep Spin Plasmid Kit (QIAGEN).

The purified plasmid was sequenced on 373A DNA Sequencer (ABI) using M13 Primer M4 and M13 Primer RV (Takara Shuzo Co., Ltd.). The plasmid that was confirmed to have the correct nucleotide sequence without any deletion was designated "C λ /pUC19".

(iii) Construction of gene encoding human L-chain κ chain C-region

A DNA fragment encoding the L-chain κ chain C-region was cloned from plasmid HEF-PM1k-gk (WO 92/19759) by PCR method. A forward primer HKAPS (SEQ ID NO: 19) was designed to contain EcoRI-, HindIII and BlnI-recognition sequences, and a backward

primer HKAPA (SEQ ID NO: 20) was designed to contain an EcoRI-recognition sequence. These primers were synthesized on 394 DNA/RNA Synthesizer (ABI).

A PCR reaction was performed using 100 μl of a reaction solution containing 0.1 μg of plasmid HEF-PM1k-gk as a template, 50 pmoles of each of primers HKAPS and HKAPA and 5U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.), over which 50 μl of mineral oil was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min., 60 ° C for 1 min. and 72° C for 1 min., thereby giving a PCR product of 360 bp. The DNA fragment was isolated and purified by electrophoresis on a 3% low-melting agarose, and then collected and purified using GENECLEAN II Kit (BIO101).

The DNA fragment thus obtained was digested with EcoRI, and then cloned into plasmid pUC19 (HindIII that had been BAP-treated. The resulting plasmid was introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant was cultured overnight in 2 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

The purified plasmid was sequenced on 373A DNA Sequencer (ABI) using M13 Primer M4 and M13 Primer RV (Takara Shuzo Co., Ltd.). The plasmid that was confirmed to have the correct nucleotide sequence was designated "C κ /pUC19".

(3) Construction of chimeric antibody L-chain expression vector

An expression vector for the chimeric #23-57-137-1 antibody L-chain was constructed. A gene encoding #23-57-137-1 L-chain V-region was ligated to the HindIII-BlnI site (located just in front of the human antibody C-region) of each of the plasmids C λ /pUC19 and C κ /pUC19, thereby obtaining pUC19 vectors that contained the DNAs encoding the chimeric #23-57-137-1 antibody L-chain V-region and either of the L-chain λ chain C-region or the L-chain κ region C-region, respectively. Each of the resulting vectors was then digested with EcoRI to separate the gene for the chimeric antibody L-chain. The gene was subcloned into HEF expression vector.

That is, a DNA fragment encoding #23-57-137-1 antibody L-chain V-region was cloned from plasmid MBC1L24 by PCR method. Primers used in the PCR method were

separately synthesized using 394 DNA/RNA Synthesizer (ABI). A backward primer MBCCHL1 (SEQ ID NO: 21) was designed to contain a HindIII-recognition sequence and a Kozak sequence (Kozak, M. et al., J. Mol. Biol. 196, 947-950, 1987), and a forward primer MBCCHL3 (SEQ ID NO: 22) was designed to contain BglII- and RcoRI-recognition sequences.

The PCR reaction was performed using 100 μl of a reaction solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 μg MBC1L24, 50 pmoles of each of primers MBCCHL1 and MBCCHL3 and 1 μl of AmpliTaq (PERKIN ELMER), over which 50 μl of mineral oil was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 45 sec., 60° C for 45 sec. and 72° C for 2 min.

A PCR product of 444 bp was electrophoresed on a 3% low-melting agarose gel, and collected and purified using GENECLEAN II Kit (BIO101). The purified PCR product was dissolved in 20 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The PCR product (1 μl) was digested in 20 μl of a reaction solution containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 8U of HindIII (Takara Shuzo Co., Ltd.) and 8U of EcoRI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The digestion solution was subjected to phenol/chloroform extraction, and the DNA of interest was collected therefrom by ethanol precipitation. The DNA was dissolved in 8 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

In the same manner, plasmid pUC19 (1 μg) was digested with HindIII and EcoRI, and subjected to phenol/chloroform extraction and then ethanol precipitation. The obtained digested plasmid was BAP-treated with alkaline phosphatase (E. coli C75; Takara Shuzo Co., Ltd.). The resulting reaction solution was extracted with phenol and chloroform, and the DNA was collected therefrom by ethanol precipitation. The DNA was dissolved in 10 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The BAP-treated plasmid pUC19 (1 μl) was ligated to the above-obtained PCR product (4 μl) using DNA Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.). The resulting plasmid was introduced into a competent cell of E. coli, JM109 (Nippon Gene Co., Ltd.), in the same manner as set forth above, to form a transformant. The transformant was plated on

2xYT agar medium containing 50 μg/ml of ampicillin and cultured at 37° C overnight. The resulting transformant was then cultured at 37° C overnight in 2 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN). After determining the nucleotide sequence, the plasmid that was confirmed to have the correct nucleotide sequence was designated "CHL/pUC19".

Each of plasmids C λ /pUC19 and C κ /pUC19 (1 μg each) was digested in 20 μl of a reaction solution containing 20 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, 8U of HindIII (Takara Shuzo Co., Ltd.) and 2U of BlnI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The digestion solution was extracted with phenol and chloroform, and the DNA was collected therefrom by ethanol precipitation. The DNA was BAP-treated at 37° C for 30 min. The reaction solution was extracted with phenol and chloroform, and the DNA was collected therefrom by ethanol precipitation. The DNA was dissolved in 10 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The plasmid CHL/pUC19 (8 μg) that contained DNA encoding #23-57-137-1 L-chain V-region was digested with HindIII and BlnI in the same manner as set forth above to give a DNA fragment of 409 bp. The DNA fragment was electrophoresed on a 3% low-melting agarose gel, and then collected and purified from the gel using GENECLEAN II Kit (BIO101). The DNA was dissolved in 10 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The DNA for L-chain V-region DNA (4 μl) was subcloned into 1 μl of each of the BAP-treated plasmids C λ /pUC19 and C κ /pUC19, and then introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant was cultured overnight in 3 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was isolated and purified using QIAprep Spin Plasmid Kit (QIAGEN). The two plasmids thus prepared were designated "MBC1L(λ)/pUC19" and "MBC1L(κ)/pUC19", respectively.

Each of plasmids MBC1L(λ)/pUC19 and MBC1L(κ)/pUC19 was digested with EcoRI and then subjected to electrophoresis on a 3% low-melting agarose gel. A DNA fragment of 743 bp was isolated and purified from the gel using GENECLEANII Kit (BIO101),

and then dissolved in 10 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

An expression vector (plasmid HEF-PM1k-gk) (2.7 μg) was digested with EcoRI and then extracted with phenol and chloroform, and the DNA was collected therefrom by ethanol precipitation. The DNA fragment was BAP-treated, and then subjected to electrophoresis on a 1% low-melting agarose gel. From the gel, a DNA fragment of 6561 bp was isolated and purified therefrom using GENECLEANII Kit (BIO101). The purified DNA fragment was dissolved in 10 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

BAP-treated HEF vector (2 μl) was ligated to an EcoRI fragment (3 μl) of each of plasmid MBC1L(λ)/pUC19 and MBC1L(κ)/pUC19. The ligation product was introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant was cultured in 2 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

The purified plasmid was digested in 20 μl of a reaction solution containing 20 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, 8U of HindIII (Takara Shuzo Co., Ltd.) and 2 U of PvuI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. This reaction gave digestion fragments of 5104/2195 bp if the fragment was inserted in the correct orientation, or gave digestion fragments of 4378/2926 bp if the fragment was inserted in the reverse orientation. The plasmid that was confirmed to have the fragment in the correct orientation was designated "MBC1L(λ)/neo" for plasmid MBC1L(λ)/pUC19 or "MBC1L(κ)/neo" for plasmid MBC1L(κ)/pUC19.

(4) Transfection of COS-7 cell

To evaluate the antigen-binding activity and the neutralizing activity of the chimeric antibodies, the expression plasmids prepared above were separately expressed transiently in a COS-7 cell.

The transient expression of the chimeric antibodies was performed using each of the combinations of plasmids MBC1HcDNA/pCOS1 and MBC1L (λ)/neo and plasmids

MBC1HcDNA/pCOS1 and MBC1L(κ)/neo, by co-tansfecting a COS-7 cell with the plasmids by electroporation using Gene Pulser (Bio Rad). That is, the plasmids (10 μ g each) were added to a COS-7 cell suspension (0.8 ml; 1 x 10^7 cells/ml) in PBS(-). The resulting solution was applied with pulses at an electrostatic capacity of 1,500V and 2 μ F to cause electroporation. After 10 min. of recovery period at room temperature, the electroporated cells were suspended in DMEM medium (GIBCO) containing 2% Ultra Low IgG fetal calf serum (GIBCO), and then cultured using a 10-cm culture dish in a CO_2 incubator. After culturing for 72 hours, a culture supernatant was collected and centrifuged to remove cell debris, and was provided for use as a sample for the subsequent ELISA. In this procedure, the purification of the chimeric antibody from the COS-7 cell culture supernatant was performed using AffiGel Protein A MAPSII Kit (Bio Rad) in accordance with the instructions included in the kit.

(5) ELISA

(i) Determination of antibody concentration

An ELISA plate for determining antibody concentration was prepared as follows. Each well of a 96-well ELISA plate (Maxisorp, NUNC) was coated with 100 μ l of a coating buffer (0.1 M $NaHCO_3$, 0.02% NaN_3) supplemented with 1 μ g/ml of goat anti-human IgG antibody (TAGO), and then blocked with 200 μ l of a dilution buffer [50 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 M NaCl, 0.05% Tween 20, 0.02% NaN_3, 1% bovine serum albumin (BSA); pH 7.2]. Each well of the plate was added with each of the serial dilutions of the COS-7 cell culture supernatant in which each of the chimeric antibodies had been expressed, or added with each of the serial dilutions of each of the chimeric antibodies per se in a purified form. The plate was incubated at room temperature for 1 hour and washed with PBS-Tween 20. Each well of the plate was then added with 100 μ l of a solution of alkaline phosphatase-conjugated goat anti-human IgG antibodies (TAGO). After the plate was incubated at room temperature for 1 hour and washed with PBS-Tween 20, each well was added with 1 mg/ml of a substrate solution ("Sigma 104", p-nitrophenylphosphoric acid, SIGMA). The solution was measured on its absorbance at 405 nm using Microplate Reader (Bio Rad) to determine the antibody

concentration. In this determination, Hu IgG1 λ Purified (The Binding Site) was used as the standard substance.

(ii) Determination of antigen-binding ability

An ELISA plate for the determination of antigen-binding ability was prepared as follows. Each well of a 96-well ELISA plate was coated with 100 μl of a coating buffer supplemented with 1 μg/ml of human PTHrP (1-34) (Peptide Research Institute), and then blocked with 200 μl of a dilution buffer. Each well was added with each of the serial dilutions of the COS-7 cell culture supernatant in which each of the chimeric antibodies had been expressed, or added with each of the serial dilutions of each of the chimeric antibodies per se in a purified form. After the plate was incubated at room temperature and washed with PBS-Tween 20, each well of the plate was added with 100 μl of a solution of alkaline phosphatase-conjugated goat anti-human IgG antibodies (TAGO). After the plate was incubated at room temperature and washed with PBS-Tween 20, each well of the plate was added with 1 mg/ml of a substrate solution ("Sigma 104", p-nitrophenylphosphoric acid, SIGMA). The solution was measured on its absorbance at 405 nm using Microplate Reader (Bio Rad).

As a result, it was found that the chimeric antibodies had an ability to bind to human PTHrP (1-34) and the cloned mouse antibody V-regions had the correct structures (FIG. 5). It was also found that there was no difference in the ability to bind to PTHrP (1-34) between the chimeric antibody with L-chain λ chain C-region and the chimeric antibody with L-chain κ chain C-region. Therefore, the humanized antibody L-chain λ chain was used for construction of the L-chain C-region of the humanized antibody.

(6) Establishment of CHO cell line capable of stable production of chimeric antibodies

To establish a cell line capable of producing the chimeric antibodies stably, the above-prepared expression plasmids were introduced into CHO cells (DXB11).

For the establishment of a cell line capable of producing the chimeric antibodies stably, either of the following combinations of the expression plasmids for CHO cell was used:

MBC1HcDNA/pCHO1 and MBC1L(λ)/neo; and MBC1HcDNA/pCHO1 and MBC1L(κ)/neo. A CHO cell was co-transfected with the plasmids by electroporation using Gene Pulser (Bio Rad) as follows. The expression vectors were separately cleaved with a restriction enzyme PvuI to give linear DNAs. The resulting DNAs were extracted with phenol and chloroform and collected by precipitation with ethanol. The plasmid DNAs thus prepared were subjected to electroporation. That is, each of the plasmid DNAs (10 μ g each) was added to 0.8 ml of a cell suspension of CHO cells in PBS(-) ($1x10^7$ cells/ml). The resulting solution was applied with pulses at an electrostatic capacity of 1,500V and 25 μ F. After 10 min. of recovery period at room temperature, the electroporated cells were suspended in MEM-α medium (GIBCO) containing 10% fetal calf serum (GIBCO). The resulting suspension was cultured using three 96-well plates (Falcon) in a CO_2 incubator. On the day following the culturing being started, the medium was replaced by a selective medium [ribonucleoside- or deoxyribonucleoside-free MEM-α medium (GIBCO) containing 10% fetal calf serum (GIBCO) and 500 mg/ml of GENETICIN (G418Sulfate; GIBCO)]. From the culture medium, cells into which the antibody gene was introduced were selected. The selective medium is replaced by a fresh one. About two weeks after the medium replacement, the cells were observed under a microscope. When a satisfactory cell growth was observed, the amount of the antibodies produced was determined by ELISA as set forth above. Among the cells, those cells which produced a larger amount of antibodies were screened.

Then, the culturing of the established cell line capable of stable production of the antibodies was scaled up in a roller bottle using ribonucleoside- or deoxyribonucleoside-free MEM medium containing 2% Ultra Low IgG fetal calf serum. On day 3 and day 4 of the culturing, the culture supernatant was collected and then filtered on a 0.2- μ m filter (Millipore) to remove cell debris therefrom.

Purification of the chimeric antibodies from the CHO cell culture supernatant was performed using POROS Protein A Column (PerSeptive Biosystems) on ConSep LC100 (Millipore) in accordance with the instructions included in the kit. The purified chimeric antibodies were provided for use as samples for the determination of neutralizing activity and for the examination of therapeutic efficacy in hypercalcemic model animals. The

concentration and the antigen-binding activity of the purified chimeric antibodies were determined using the same ELISA system as set forth above.

[REFERENCE EXAMPLE 4] Construction of humanized antibody

(1) Construction of humanized antibody H-chain

(i) Construction of humanized H-chain V-region

A humanized #23-57-137-1 antibody H-chain was produced by CDR-grafting technique by means of PCR method. For the production of a humanized #23-57-137-1 antibody H-chain (version "a") having FRs derived from human antibody S31679 (NBRF-PDB; Cuisinier, A. M. et al., Eur. J. Immunol., 23, 110-118, 1993), the following six PCR primers were used: CDR-grafting primers: MBC1HGP1 (SEQ ID NO: 23) and MBC1HGP3 (SEQ ID NO: 24) (both containing a sense DNA sequence) and MBC1HGP2 (SEQ ID NO: 25) and MBC1HGP4 (SEQ ID NO: 26) (both containing an antisense DNA sequence), all of which containing a 15-21 bp complementary sequence on both terminal ends thereof; and external primers: MBC1HVS1 (SEQ ID NO: 27) and MBC1HVR1 (SEQ ID NO: 28) having a homology to the CDR-grafting primers MBC1HGP1 and MBC1HGP4, respectively.

The CDR-grafting primers MBC1HGP1, MBC1HGP2, MBC1HGP3 and MBC1HGP4 were separated on an urea-denatured polyacrylamide gel (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989), and extracted therefrom by crush-and-soak method (Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989) in the following manner.

Each of the CDR-grafting primers (1 nmole) was separated on a 6% denatured polyacrylamide gel to give DNA fragments. From the resulting DNA fragments, a DNA fragment having a desired length was identified on a silica gel thin plate by irradiation of UV ray and then collected therefrom by crush-and-soak method. The resulting DNA was dissolved in 20 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.). The PCR reaction solution (100 μl) comprised 1 μl of each of the above-mentioned CDR-grafting

primers MBC1HGP1, MBC1HGP2, MBC1HGP3 and MBC1HGP4, 0.25 mM dNTPs and 2.5U of TaKaRa Ex Taq in the buffer. The PCR reaction was run for 5 cycles under the conditions: 94° C for 1 min., 55° C for 1 min. and 72° C for 1 min. The resulting reaction solution was added with the external primers MBC1HVS1 and MBC1HVR1 (50 pmoles each). Using this reaction mixture, the PCR reaction was run for additional 30 cycles under the same conditions. The DNA fragment thus amplified was separated by agarose gel electrophoresis on a 4% Nu Sieve GTG agarose (FMC Bio. Products).

An agarose segment containing a DNA fragment of 421 bp was excised, and the DNA fragment was purified therefrom using GENECLEANII Kit (BIO101) in accordance with the instructions included in the kit. The DNA fragment thus purified was precipitated with ethanol and then dissolved in 20 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The resulting PCR reaction mixture was used for subcloning of the DNA fragment into plasmid pUC19 that had been digested with BamHI and HindIII, and subsequently the nucleotide sequence of the resulting plasmid was determined. A plasmid having the correct nucleotide sequence was designated "hMBCHv/pUC19".

(ii) Construction of H-chain V-region of Humanized H-chain cDNA

To ligate to cDNA for humanized H-chain C-region C γ 1, the DNA for the humanized H-chain V-region constructed in the above step was modified by PCR method. For the PCR method, a backward primer MBC1HVS2 was designed to hybridize to the sequence encoding the 5' region of the leader sequence for the V-region and to have a Kozak consensus sequence (Kozak et al., J. Mol. Biol. 196, 947-950, 1987) and HindIII- and EcoRI-recognition sequences; and a forward primer MBC1HVR2 was designed to hybridize to both the DNA sequence encoding the 3' region of the J region and the DNA sequence encoding the 5' region of the C-region and to have ApaI- and SmaI-recognition sequences.

The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and a buffer appended thereto. The PCR reaction solution comprised 0.4 μg of hMBCHv/pUC19 as a DNA template, 50 pmoles of each of MBC1HVS2 and MBC1HVR2 as primers, 2.5U of TaKaRa Ex Taq and 0.25 mM dNTPs in the buffer. The PCR reaction was

run for 30 cycles under the conditions: 94° C for 1 min., 55° C for 1 min. and 72° C for 1 min. The DNA fragment thus amplified was separated by agarose gel electrophoresis on a 3% Nu Sieve GTG agarose (FMC Bio. Products).

A gel segment containing a DNA fragment of 456 bp was excised, and the DNA fragment was purified therefrom using GENECLEANII Kit (BIO101) in accordance with the instructions included in the kit. The DNA fragment thus purified was precipitated with ethanol and then dissolved in 20 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. The PCR reaction solution thus obtained was used for subcloning of the DNA fragment into plasmid pUC19 that had been digested with EcoRI and SmaI, and then the resulting plasmid was sequenced. As a result, a plasmid was obtained which contained a DNA encoding mouse H-chain V-region derived from hybridoma #23-57-137-1 and also contained EcoRI- and HindIII-recognition sequences and a Kozak sequence on the 5' region and ApaI- and SmaI-recognition sequences on the 3' region, which was designated "hMBC1Hv/pUC19".

(2) Construction of expression vector for humanized antibody H-chain

Plasmid RVh-PM1f-cDNA carrying a cDNA sequence for hPM1 antibody H-chain was digested with ApaI and BamHI to give a DNA fragment containing a DNA fragment containing a DNA encoding the H-chain C-region. The DNA fragment was introduced into plasmid hMBC1Hv/pUC19 that had been digested with ApaI and BamHI. The obtained plasmid was designated "hMBC1HcDNA/pUC19". This plasmid contained both a DNA encoding the humanized #23-57-137-1 antibody H-chain V-region and a DNA encoding the human H-chain C-region C γ 1 and had EcoRI- and HindIII-recognition sequences on the 5' region and a BamHI-recognition sequence on the 3' region. The nucleotide sequence and the corresponding amino acid sequence of the humanized H-chain version "a" carried on the plasmid hMBC1HcDNA/pUC19 are shown in SEQ ID NO: 58 and SEQ ID NO: 56, respectively.

The plasmid hMBC1HcDNA/pUC19 was digested with EcoRI and BamHI to give a DNA fragment containing a DNA encoding the H-chain. The DNA fragment was introduced

into expression plasmid pCOS1 that had been digested with EcoRI and BamHI. As a result, an expression plasmid for a humanized antibody was obtained, which was designated "hMBC1HcDNA/pCOS1".

To produce a plasmid used for expression in a CHO cell, plasmid hMBC1HcDNA/pUC19 was digested with EcoRI and BamHI to give a DNA fragment containing a DNA encoding the H-chain. The DNA fragment was introduced into expression vector pCHO1 that had been digested with EcoRI and BamHI. As a result, an expression plasmid for the humanized antibody was obtained, which was designated "hMBC1HcDNA/pCHO1".

(3) Construction of L-chain hybrid V-region

(i) Preparation of FR1,2/FR3,4 hybrid antibody

A gene for the FR hybrid L-chain having both FRs from a humanized antibody and FRs from a mouse (chimeric) antibody was constructed, and evaluated each region for the humanization. In this step, a hybrid antibody having FR1 and FR2 both derived from a human antibody and FR3 and FR4 both derived from a mouse antibody was prepared by utilizing the AflII restriction site located on CDR2.

Plasmids MBC1L(λ)/neo and hMBC1L(λ)/neo (10 μg each) were separately digested in 100 μl of a reaction solution containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 0.01% (w/v) of BSA and 10 U of AflII (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The reaction solutions were subjected to electrophoresis on a 2% low-melting agarose gel, thereby giving DNA fragments of 6282 bp (referred to as "c1") and 1022 bp (referred to as "c2") from the plasmid MBC1L(λ)/neo or DNA fragments of 6282 bp (referred to as "h1") and 1022 bp (referred to as "h2") from the plasmid hMBC1L(λ)/neo. These DNA fragments were collected and purified from the gels using GENECLEANII Kit (BIO101).

Each of the c1 and h1 fragments (1 μg each) was BAP-treated. The DNA fragment was extracted with phenol and chloroform, collected by ethanol precipitation, and then dissolved in 10 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The BAP-treated c1 and h1 DNA fragments (1 μl each) were ligated to the h2 and c2 DNA fragments (4 μl each), respectively, (at 4° C overnight). Each of the ligation products was introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant was cultured in 2 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

The purified plasmid was digested in 20 μl of a reaction solution containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and either 2U of ApaLI (Takara Shuzo Co., Ltd.) or 8U of BamHI (Takara Shuzo Co., Ltd.) and HindIII (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. It was expected that if the c1-h2 was ligated correctly, this digestion reaction would give fragments of 5560/1246/498 bp (by the ApaLI digestion) or fragments of 7134/269 bp (by the BamHI/HindIII digestion). Based on this expectation, the desired plasmids were identified.

The expression vector encoding the human FR1,2/mouse FR3,4 hybrid antibody L-chain was designated "h/mMBC1L(λ)/neo". On the other hand, since a clone for the h1-c1 could not be obtained, recombination on a pUC vector was performed and then the resulting recombinant product was cloned into a HEF vector. In this procedure, plasmid hMBC1La λ /pUC19, which contained DNA encoding a humanized antibody L-chain V-region without any amino acid replacements, and plasmid hMBC1Ld λ /pUC19, which contained a DNA encoding a humanized antibody L-chain V-region with an amino acid replacement at the 91-position amino acid tyrosine in FR3 (i.e., the 87th amino acid in accordance with The Kabat's prescription) by isoleucine, were used as templates.

Plasmids MBC1L(λ)/pUC19, hMBC1La λ /pUC19 and hMBC1Ld λ /pUC19 (10 μl each) were separately digested in 30 μl of a reaction solution containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 0.01% (w/v) of BSA, 16U of HindIII and 4U of AflII at 37° C for 1 hour. The reaction solutions were separately subjected to electrophoresis on a 2% low-melting agarose gel, thereby giving a DNA fragment of 215 bp from plasmid MBC1L(λ)/pUC19 (referred to as "c2'") and a DNA fragment of 3218 bp from each of plasmids hMBC1La λ /pUC19 and hMBC1Ld λ /pUC19 (referred to as "ha1'" and "hd1'", respectively). These DNA fragments were collected and purified using

GENECLEANII Kit (BIO101).

Each of the ha1' and hd1' fragments was ligated to the c2' fragment and then introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant was cultured in 2 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN). The plasmids thus prepared were designated "m/hMBC1La λ /pUC19" for the ha1' fragment-containing plasmid and "m/hMBC1Ld λ /pUC19" for the hd1' fragment-containing plasmid.

Each of the plasmids m/hMBC1La λ /pUC19 and m/hMBC1Ld λ /pUC19 was digested with EcoRI. The DNA fragment of 743 bp was electrophoresed on a 2% low-melting agarose gel, and then collected and purified therefrom using GENECLEANII Kit (BIO101). The resulting DNA fragment was dissolved in 20 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

Each of the DNA fragments (4 μl each) was ligated to the above-obtained BAP-treated HEF vector (1 μl). The ligation product was introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant was cultured in 2 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit (QIAGEN).

Each of the purified plasmids was digested in 20 μl of a reaction solution containing 20 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, 8U of HindIII (Takara Shuzo Co., Ltd.) and 2U of PvuI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. It was expected that if the DNA fragment was inserted in the plasmid in a correct orientation, this digestion would give digestion fragments of 5104/2195 bp, whereas if the DNA fragment is inserted in the plasmid in the reverse orientation, this digestion would give digestion fragments of 4378/2926 bp. The plasmid DNA was identified based on the expectation. The plasmids thus obtained were expression vectors encoding mouse FR1,2/human FR3,4 hybrid antibody L-chain, which were designated expression vectors "m/hMBC1La λ /neo" and "m/hMBC1Ld λ / neo", respectively.

(ii) Preparation of FR1/FR2 hybrid antibody

An FR1/FR2 hybrid antibody was prepared in the same manner as set forth above utilizing a SnaBI restriction site located on CDR1.

Plasmids MBC1L(λ)/neo and h/mMBC1L(λ)/neo (10 μg each) were separately digested in 20 μl of a reaction solution containing 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 0.01% (w/v) of BSA and 6U of SnaBI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour. The resulting reaction solutions were further digested in 50 μl of a reaction solution containing 20 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, 0.01% (w/v) of BSA and 6U of PvuI at 37° C for 1 hour.

The resulting reaction solutions were separately subjected to electrophoresis on a 1.5% low-melting agarose gel, thereby giving DNA fragments of 4955 bp (m1) and 2349 bp (m2) from the plasmid MBC1L(λ)/neo and DNA fragments of 4955 bp (hm1) and 2349 bp (hm2) from the plasmid h/mMBC1L(λ)/neo. These DNA fragments were collected and purified from the gels using GENECLEANII Kit (BIO101). Each of the DNA fragments obtained was dissolved in 40 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The m1 and hm1 fragments (1 μl each) were ligated to the hm2 and m2 fragments (4 μl each), respectively. Each of the resulting ligation products was introduced into a competent cell of E. coli, JM109, to form a transformant. The transformant obtained was cultured in 2 ml of 2xYT medium containing 50 μg/ml of ampicillin. From the cell fraction, the plasmid was purified using QIAprep Spin Plasmid Kit QIAGEN).

Each of the purified plasmids was digested in 20 μl of a reaction solution containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT and either 8U of ApaI (Takara Shuzo Co., Ltd.) or 2U of ApaLI (Takara Shuzo Co., Ltd.) at 37° C for 1 hour.

It was expected that if the fragments were ligated correctly, the digestion reaction would give a fragment of 7304 bp (by the ApaI digestion) or fragments of 5560/1246/498 bp (by the ApaLI digestion) for m1-hm2, and would give fragments of 6538/766 bp (by the ApaI digestion) or fragments of 3535/2025/1246/498 bp (by the ApaLI digestion) for hm1-m2. Based on this expectation, the plasmids were identified. As a result, an expression vector encoding a human FR1/mouse FR2,3,4 hybrid antibody L-chain (designated "hmmMBC1L(λ

)/neo") and an expression vector encoding a mouse FR1/human FR2/mouse FR3,4 hybrid antibody L-chain (designated "mhmMBC1L(λ)/neo") were obtained.

(4) Construction of humanized antibody L-chain

A humanized #23-57-137-1 antibody L-chain was prepared by CDR-grafting technique by means of PCR method. For the preparation of a humanized #23-57-137-1 antibody L-chain (version "a") that contained FR1, FR2 and FR3 derived from human antibody HSU03868 (GEN-BANK, Deftos M. et al., Scand. J. Immunol., 39, 95-103, 1994) and FR4 derived from human antibody S25755 (NBRF-PDB), six PCR primers were used.

The six primers were as follows: CDR-grafting primers MBC1LGP1 (SEQ ID NO: 29) and MBC1LGP3 (SEQ ID NO: 30), both having a sense DNA sequence, CDR-grafting primers MBC1LGP2 (SEQ ID NO: 31) and MBC1LGP4 (SEQ ID NO: 32), both having an antisense DNA sequence, all of which had a 15-21 bp complementary sequence on the both terminal ends; and external primers MBC1LVS1 (SEQ ID NO: 33) and MBC1LVR1 (SEQ ID NO: 34) having a homology to the CDR-grafting primers MBC1LGP1 and MBC1LGP4, respectively.

The CDR-grafting primers MBC1LGP1, MBC1LGP2, MBC1LGP3 and MBC1LGP4 were separated on a urea-denatured polyacrylamide gel (Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989) and extracted therefrom by crush-and-soak method (Molecular Cloning: A Laboratory Manual, Sambrook et al., Cold Spring Harbor Laboratory Press, 1989).

Each of the CDR-grafting primers (1 nmole each) was separated on a 6% denatured polyacrylamide gel. The identification of the DNA fragment of a desired length was performed on a silica gel thin plate by irradiation of UV ray. The desired DNA fragment was collected from the gel by crush-and-soak method. The collected DNA fragment was dissolved in 20 μl of a solution containing 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and a buffer appended thereto. The PCR reaction solution comprised (per 100 μl) 1 μl of each of the CDR-grafting primers MBC1LGP1, MBC1LGP2, MBC1LGP3 and MBC1LGP4, 0.25

mM dNTPs, 2.5U of TaKaRa Ex Taq in the buffer. The PCR reaction was run for 5 cycles under the conditions: 94° C for 1 min., 55° C for 1 min. and 72° C for 1 min. The resulting reaction mixture was added with 50 pmoles of each of the external primers MBC1LVS1 and MBC1LVR1. Using this reaction mixture, the PCR reaction was run for additional 30 cycles under the same conditions. The DNA fragment thus amplified was separated by agarose gel electrophoresis on a 3% Nu Sieve GTG agarose (FMC Bio. Products).

An agarose segment containing a DNA fragment of 421 bp was excised, and the DNA fragment was purified therefrom using GENECLEANII Kit (BIO101) in accordance with the instructions included in the kit. The PCR reaction mixture thus obtained was used for subcloning of the DNA fragment into plasmid pUC19 that had been digested with BamHI and HindIII. The resulting plasmid was sequenced. The plasmid thus prepared was designated "hMBCL/pUC19". In this plasmid, however, the 104-position amino acid (corresponding to the 96th amino acid in accordance with the Kabat's prescription) of CDR4 was replaced by arginine. For the correction of this amino acid to tyrosine, a correction primer MBC1LGP10R (SEQ ID NO: 35) was designed and synthesized. The PCR reaction was performed using TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and a buffer appended thereto. The PCR reaction solution comprised (per 100 μl) 0.6 μg of the plasmid hMBCL/pUC19 as a template DNA, 50 pmoles of each of the primers MBC1LVS1 and MBC1LGP10R, 2.5U of TaKaRa Ex Taq (Takara Shuzo Co., Ltd.) and 0.25 mM dNTPs in the buffer, over which mineral oil (50 μl) was layered. The PCR reaction was run for 30 cycles under the conditions: 94° C for 1 min., 55° C for 1 min. and 72° C for 1 min. The DNA fragment thus amplified was separated by agarose gel electrophoresis on a 3% Nu Sieve GTG agarose (FMC Bio. Products).

A gel segment containing a DNA fragment of 421 bp was excised, and the DNA fragment was purified therefrom using GENECLEANII Kit (BIO101) in accordance with the instructions included in the kit. The PCR reaction mixture thus prepared was used for subcloning of the DNA fragment into plasmid pUC19 that had been digested with BamHI and HindIII.

The plasmid was sequenced using M13 Primer M4 and M13 Primer RV. As a result,

it was confirmed that the plasmid had the correct sequence. The plasmid was then digested with HindIII and BlnI, and a DNA fragment of 416 bp was separated by electrophoresis on a 1% agarose gel. The DNA fragment was purified using GENECLEANII Kit (BIO101) in accordance with the instructions included in the kit, and then introduced into plasmid C λ /pUC19 that had been digested with HindIII and BlnI. The resulting plasmid was designated "hMBC1La λ /pUC19". This plasmid was digested with EcoRI to give a DNA fragment encoding humanized L-chain. The DNA fragment was introduced into plasmid pCOS1 so that the initiation codon for the humanized L-chain was located downstream to the EF1 α promoter. The plasmid thus obtained was designated "hMBC1La λ /pCOS1". The DNA sequence (including the corresponding amino acid sequence) of the humanized L-chain version "a" is shown in SEQ ID NO: 66. The amino acid sequence of the version "a" is also shown in SEQ ID NO: 47.

A humanized L-chain version "b" was prepared using mutagenesis by PCR method. The version "b" was designed such that the 43-position amino acid glycine (corresponding to the 43th amino acid in accordance with the Kabat's prescription) was replaced by proline and the 49-position amino acid lysine (corresponding to the 49th amino acid accordance with the Kabat's prescription) by aspartic acid in the version "a". The PCR reaction was performed using plasmid hMBC1La λ /pUC19 as a template and a mutagenic primer MBC1LGP5R (SEQ ID NO: 36) and a primer MBC1LVS1. The DNA fragment obtained was digested with BamHI and HindIII, and the digestion fragment was subcloned into the BamHI-HindIII site of pUC19. After sequencing, the plasmid was digested with HindIII and Afl II, and the resulting digestion fragment was ligated to plasmid hMBC1La λ /pUC19 that had been digested with HindIII and AflII.

The plasmid thus obtained was designated "hMBC1Lb λ /pUC19". This plasmid was digested with EcoRI to give a DNA fragment containing a DNA encoding the humanized L-chain. The DNA fragment was introduced into plasmid pCOS1 such that the initiation codon for the humanized L-chain was located downstream to the EF1 α promoter. The plasmid thus obtained was designated "hMBC1Lb λ /pCOS1".

A humanized L-chain version "c" was prepared using mutagenesis by PCR method.

The version "c" was designed such that the 84-position amino acid serine (corresponding to the 80th amino acid in accordance with the Kabat's prescription) was replaced by proline. The PCR reaction was performed using plasmid hMBC1La λ /pUC19 as a template and a mutagenic primer MBC1LGP6S (SEQ ID NO: 37) and a primer M13 Primer RV. The DNA fragment obtained was digested with BamHI and HindIII and then subcloned into pUC19 that had been digested with BamHI and HindIII.

After sequencing, the plasmid was digested with BstPI and Aor51HI, and the resulting DNA fragment was ligated to plasmid hMBC1La λ /pUC19 that had been digested with BstPI and Aor51HI. The plasmid thus obtained was designated "hMBC1Lc λ /pUC19". This plasmid was digested with EcoRI to give a DNA fragment containing a DNA encoding the humanized L-chain. The fragment was introduced into the EcoRI site of plasmid pCOS1 such that the initiation codon for the humanized L-chain was located downstream to the EF1 α promoter. The plasmid thus obtained was designated "hMBC1Lc λ /pCOS1".

Humanized L-chain versions "d", "e" and "f" were also prepared using mutagenesis by PCR method. The versions "d", "e" and "f" were designed such that the 91-position amino acid tyrosine (corresponding to the 87th amino acid in accordance with the Kabat's prescription) was replaced by isoleucine in the versions "a", "b" and "c", respectively. For each of the versions "d", "e" and "f", a PCR reaction was performed using each of plasmid hMBC1La λ /pCOS1 (for version "d"), hMBC1Lb λ /pCOS1 (for version "e") and hMBC1Lc λ /pCOS1 (for version "f"), respectively, as a template, a mutagenic primer MBC1LGP11R (SEQ ID NO: 38) and a primer M-S1 (SEQ ID NO: 44). The DNA fragment thus obtained was digested with BamHI and HindIII and then subcloned into pUC19 that had been digested with BamHI and HindIII. After sequencing, the plasmid was digested with HindIII and BlnI, and the resulting digestion fragment was ligated to plasmid C λ /pUC19 that had been digested with HindIII and BlnI.

The plasmids thus obtained were respectively designated "hMBC1Ld λ /pUC19" (for version "d"), "hMBC1Le λ /pUC19" (for version "e") and "hMBC1Lf λ /pUC19" (for version "f"). Each of these plasmids was digested with EcoRI to give a DNA fragment containing a DNA encoding the humanized L-chain. The DNA fragment was introduced into the EcoRI

site of plasmid pCOS1 such that the initiation codon for the humanized L-chain was located downstream to the EF1 α promoter of the plasmid. The plasmids thus obtained were respectively designated "hMBC1Ld λ /pCOS1" (for version "d"), "hMBC1Le λ /pCOS1" (for version "e") and "hMBC1Lf λ /pCOS1" (for version "f").

Humanized L-chain versions "g" and "h" were also prepared using mutagenesis by PCR method. The versions "g" and "h" were designed such that the 36-position amino acid histidine (corresponding to the 36th amino acid in accordance with the Kabat's prescription) was replaced by tyrosine in the versions "a" and "d", respectively. The PCR reaction was performed using a mutagenic primer MBC1LGP9R (SEQ ID NO: 39), M13 Primer RV and plasmid hMBC1La λ /pUC19 as a template. An additional PCR was performed using the PCR product thus obtained and M13 Primer M4 as primers and plasmid hMBC1La λ /pUC19 as a template. The DNA fragment obtained was digested with HindIII and BlnI and then subcloned into plasmid C λ /pUC19 that had been digested with HindIII and BlnI. Using this plasmid as a template, a PCR reaction was performed using primers MBC1LGP13R (SEQ ID NO: 40) and MBC1LVS1. The PCR fragment obtained was digested with ApaI and HindIII and then introduced into either of plasmids hMBC1La λ /pUC19 and hMBC1Ld λ /pUC19 that had been digested with ApaI and HindIII. The plasmids obtained were sequenced. Plasmids that were confirmed to contain the correct sequence were designated "hMBC1Lg λ /pUC19" (for version "g") and "hMBC1Lh λ /pUC19" (for version "h"). Each of these plasmids was digested with EcoRI to give a DNA fragment containing a DNA encoding the humanized L-chain. The DNA fragment was introduced into the EcoRI site of plasmid pCOS1 such that the initiation codon for the humanized L-chain was located downstream to the EF1 α promoter. The plasmids thus obtained were respectively designated "hMBC1Lg λ /pCOS1" (for version "g") and "hMBC1Lh λ /pCOS1" (for version "h").

Humanized L-chain versions "i", "j", "k", "l", "m", "n" and "o" were also prepared using mutagenesis by PCR method. The PCR reaction was performed using plasmid hMBC1La λ /pUC19 as a template and a mutagenic primer MBC1LGP14S (SEQ ID NO: 41) and a primer V1RV (λ) (SEQ ID NO: 43). The resulting DNA fragment was digested with ApaI and BlnI and then subcloned into plasmid hMBC1Lg λ /pUC19 that had been digested

with ApaI and BlnI. The plasmid obtained was sequenced, and the clone into which the mutation for each version was introduced was selected. The plasmid thus obtained was designated "hMBC1Lx λ /pUC19 (x=i, j, k, l, m, n or o)". This plasmid was digested with EcoRI to give a DNA fragment containing a DNA encoding the humanized L-chain. The DNA fragment was introduced into the EcoRI site of plasmid pCOS1 such that the initiation codon for the humanized L-chain was located downstream to the EF1 α promoter. The plasmid thus obtained was designated "hMBC1Lx λ /pCOS1" (x = i, j, k, l, m, n or o). The DNA sequences (including the corresponding amino acid sequences) of the versions "j", "l", "m" and "o" are shown in SEQ ID NOs: 67, 68, 69 and 70, respectively. The amino acid sequences of these versions are also shown in SEQ ID Nos: 48, 49, 50 and 51, respectively.

Humanized L-chain versions "p", "q", "r", "s" and "t" were designed such that the 87-position amino acid (tyrosine) was replaced by isoleucine in the versions "i", "j", "m", "l" and "o", respectively. These versions were prepared utilizing an Aor51MI restriction site on FR3 and replacing that site of each of the versions "i", "j", "m", "l" or "o" by that site of the version "h". That is, an Aor51HI restriction fragment (514 bp) containing CDR3, a part of FR3 and the entire FR4 were removed from an expression plasmid hMBC1Lx λ /pCOS1 (x = i, j, m, l or o). To the removed site, an Aor51HI restriction fragment (514 bp) in the expression plasmid hMBC1Lh λ /pCOS, which containing CDR3 and a part of FR3 and the entire FR4, was ligated, so that the 91-position amino acid tyrosine (corresponding to the 87th amino acid in accordance with the Kabat's prescription) was replaced by isoleucine. The resulting plasmid was sequenced. A clone of each of the versions "i", "j", "m" "l" and "o" in which 91-position amino acid tyrosine (corresponding to the 87th amino acid in accordance with the Kabat's prescription) was replaced by isoleucine was selected. These modified versions respectively corresponding to the versions "i", "j", "m" "l" and "o" were designated versions "p", "q", "s", "r" and "t", respectively. The obtained plasmid was designated "hMBC1Lx λ /pCOS1 (x =p, q, s, r or t). The DNA sequences (including the corresponding amino acids) of the versions "q", "r", "s" and "t" are shown in SEQ ID Nos: 71, 72, 73 and 74, respectively. The amino acid sequences of these versions are also shown in SEQ ID Nos: 52, 53, 54 and 55, respectively.

Plasmid hMBC1Lq λ /pCOS1 was digested with HindIII and EcoRI and then subcloned into plasmid pUC19 that had been digested with HindIII and EcoRI. The plasmid thus obtained was designated "hMBC1Lq λ /pUC19.

The positions of the replaced amino acids in the individual versions of the humanized L-chain are shown in Table 2 below.

Table 2

Positions of replaced amino acid in sequence listings
(amino acid numbering in accordance with the Kabat's prescription)

Versions	36	43	45	47	49	80	87
a							
b		P			D		
c						P	
d							I
e		P			D		I
f						P	I
g	Y						
h	Y						I
i	Y		K				
j	Y		K		D		
k	Y		K	V			
l	Y		K	V	D		
m	Y				D		
n	Y			V			
o	Y			V	D		
p	Y		K				I
q	Y		K		D		I
r	Y				D		I
s	Y		K	V	D		I
t	Y			V	D		I

In Table 2, capital letters represent the following amino acids: Y: tyrosine; P: proline; K: lysine, V: valine; D: aspartic acid; and I: isoleucine.

E. coli strains each containing plasmids hMBC1HcDNA/pUC19 and hMBC1Lq λ /pUC19 were designated "Escherichia coli JM109 (hMBC1HcDNA/pUC19)" and "Escherichia coli JM109 (hMBC1Lq λ /pUC19)", respectively, which have been deposited under the terms of Budapest Treaty at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki, Japan) on August 15, 1996, under the accession No. FERM BP-5629 for Escherichia coli JM109

(hMBC1HcDNA/pUC19), and FERM BP-5630 for Escherichia coli JM109 (hMBC1Lq λ /pUC19).

(5) Transfection into COS-7 cell

For the evaluation of the antigen-binding activity and the neutralizing activity of the hybrid antibodies and the humanized #23-57-137-1 antibodies, the above-prepared expression plasmids were expressed transiently in COS-7 cells. For the transient expression of the L-chain hybrid antibodies, each of the following combinations of plasmids were co-transfected into a COS-7 cell by electroporation using Gene Pulser (Bio Rad): hMBC1HcDNA/pCOS1 and h/mMBC1L(λ)/neo; hMBC1HcDNA/pCOS1 and m/hMBC1La λ /neo; hMBC1HcDNA/pCOS1 and m/hMBC1Ld λ /neo; hMBC1HcDNA/pCOS1 and hmmMBC1L(λ)/neo; and hMBC1HcDNA/pCOS1 and mhmMBC1L(λ)/neo. That is, a cell suspension (0.8 ml) of COS-7 cells in PBS(-) ($1x10^7$ cells/ml) was added with each combination of the plasmid DNAs (10 μg each). The resulting solution was applied with pulses at an electrostatic capacity of 1,500V and 25 μF. After 10 min. of recovery period at room temperature, the electroporated cells were suspended in DMEM medium containing 2% Ultra Low IgG fetal calf serum (GIBCO), and then cultured using a 10-cm culture dish in a CO_2 incubator. After culturing for 72 hours, a culture supernatant was collected and centrifuged to remove cell debris. The solutions thus prepared were provided for use in the ELISA below.

For the transient expression of the humanized #23-57-137-1 antibodies, plasmids of hMBC1HcDNA/pCOS1 and hMBC1Lx λ /pCOS1 (x = a-t) were co-transfected into a COS-7 cell using Gene Pulser (Bio Rad) in the same manner as described for the hybrid antibodies above. The culture supernatants were prepared and provided for use in the ELISA below.

The purification of the hybrid antibodies and the humanized antibodies from the COS-7 cell culture supernatants was performed using AffiGel Protein A MAPSII Kit (Bio Rad) in accordance with the instructions included in the kit.

(6) ELISA

(i) Determination of antibody concentration

An ELISA plate for determining antibody concentration was prepared as follows. Each well of a 96-well ELISA plate (Maxisorp, NUNC) was coated with 100 μl of a coating buffer (0.1 M $NaHCO_3$, 0.02% NaN_3) containing 1 μg/ml of goat anti-human IgG antibody (TAGO) and then blocked with 200 μl of a dilution buffer [50 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 M NaCl, 0.05% Tween 20, 0.02% NaN_3, 1% bovine serum albumin (BSA); pH 7.2]. Each of the wells was added with each of the serial dilutions of the COS cell culture supernatant in which each of the hybrid antibodies and the humanized antibodies was expressed, or added with each of the serial dilutions of each of the hybrid antibodies and humanized antibodies in a purified form. The plate was incubated at room temperature for 1 hour and washed with PBS-Tween 20. Subsequently, each of the wells was added with 100 μl of alkaline phosphatase-conjugated goat anti-human IgG antibody (TAGO). The plate was incubated at room temperature for 1 hour and washed with PBS-Tween 20. Subsequently, each of the wells was added with 1 mg/ml of a substrate solution ("Sigma 104", p-nitrophenylphosphoric acid, SIGMA). The solution in each well was measured on its absorbance at 405 nm using Microplate Reader (Bio Rad) to determine the antibody concentration. In this determination, Hu IgG1 λ Purified (The Binding Site) was used as the standard substance.

(ii) Determination of antigen-binding ability

An ELISA plate for determining antigen-binding ability was prepared as follows. Each well of a 96-well ELISA plate (Maxisorp, NUNC) was coated with 100 μl of a coating buffer containing 1 μg/ml of human PTHrP (1-34) and then blocked with 200 μl of a dilution buffer. Subsequently, each well was added with each of the serial dilutions of the COS-7 cell culture supernatant in which each of the hybrid antibodies and humanized antibodies was expressed, or added with each of the serial dilutions of each of the hybrid antibodies and humanized antibodies in a purified form. The plate was incubated at room temperature and washed with PBS-Tween 20. Subsequently, each well was added with 100 μl of alkaline phosphatase-conjugated goat anti-human IgG antibody (TAGO). The plate was incubated at room temperature and washed with PBS-Tween 20. Subsequently, each well was added with 1 mg/ml of a substrate solution ("Sigma 104", p-nitrophenylphosphoric acid,

SIGMA). The solution was measured on its absorbance at 405 nm using Microplate Reader (Bio Rad).

(7) Confirmation of activities

(i) Evaluation of humanized H-chain

It was found that an antibody having both a humanized H-chain version "a" and a chimeric L-chain exhibited the same level of PTHrP-binding activity as that of a chimeric antibody. This result suggests that the version "a" achieves the humanization of the H-chain V-region in the degree enough to evaluate the humanization. Therefore, the humanized H-chain version "a" was provided for use as a humanized antibody H-chain in the following experiments.

(ii) Activity of hybrid antibodies

(ii-a) FR1,2/FR3,4 hybrid antibody

When the L-chain was h/mMBC1L(λ), no antigen-binding activity was observed. In contrast, when the L-chain was either m/hMBC1La λ or m/hMBC1Ld λ , the same level of antigen-binding activity as that of the chimeric #23-57-137-1 antibody was observed (FIG. 7). These results suggest that FR3 and FR4 have no problem as humanized antibodies but FR1 and FR2 contain amino acid residue(s) that need to be replaced.

(ii-b) FR1/FR2 hybrid antibody

When the L-chain was mhmMBC1L (λ), no antigen-binding activity was observed. In contrast, when the L-chain was hmmMBC1L(λ), the same level of antigen-binding activity as that of the chimeric #23-57-137-1 antibody was observed (FIG. 8). These results suggest that FR1 has no problem as a humanized antibody but FR2 contains amino acid residue(s) that need to be replaced.

(iii) Activity of humanized antibodies

The antigen-binding activity of the humanized antibodies having the L-chain versions

"a" to "t", respectively, were determined. As a result, it was found that the humanized antibodies having the L-chain versions "j", "l" "m", "o", "q", "r", "s" and "t" exhibited the same levels of PTHrP-binding activity as that of the chimeric antibody.

(8) Establishment of CHO cell line capable of stable production of antibody

For establishing a cell line capable of stable production of humanized antibodies, each of the above-prepared expression plasmids was introduced into a CHO cell (DXB11).

That is, the establishment of a cell line capable of stable production of a humanized antibody was performed using each of the following combinations of plasmids as expression vectors for a CHO cell; hMBC1HcDNA/pCHO1 and hMBC1Lm λ /pCOS1; hMBC1HcDNA/pCHO1 and hMBC1Lq λ /pCOS1; and hMBC1HcDNA/pCHO1 and hMBC1Lr λ /pCOS1. The plasmids were co-transfected into a CHO cell by electroporation using Gene Pulser (Bio Rad). Subsequently, the expression vectors were separately cleaved with restriction enzyme PvuI to give linear DNA fragments. The resulting DNA fragments were extracted with phenol and chloroform and then precipitated with ethanol. The DNA fragments thus prepared were used in the subsequent electroporation. That is, the plasmid DNA fragments (10 μg each) were added to 0.8 ml of a cell suspension of CHO cells in PBS(-) ($1x10^7$ cells/ml). The resulting solution was applied with pulses at an electrostatic capacity of 1,500V and 25 μF. After 10 min. of recovery period at room temperature, the cells thus treated were suspended in MEM-α medium (GIBCO) containing 10% fetal calf serum (GIBCO), and then cultured in a CO_2 incubator using 96-well plates (Falcon). On the day following the culturing being started, the medium was replaced by ribonucleoside- or deoxyribonucleoside-free MEM-α selective medium containing 10% fetal calf serum (GIBCO) and 500 mg/ml of GENETICIN (G418Sulfate; GIBCO). From the culture medium, cells into which the antibody gene was introduced were selected. The culture medium was replaced by a fresh one. About two weeks after the medium replacement, the cells were observed microscopically. When a satisfactory cell growth was observed, the amount of the antibodies produced was determined by conventional ELISA for determination of antibody concentration as set forth above. Among the cells, those cells which produced a larger

amount of antibodies were screened.

The culturing of the established cell line capable of stable production of antibodies was scaled up in a roller bottle using a ribonucleoside- or deoxyribonucleoside-free MEM- α medium containing 2% Ultra Low IgG fetal calf serum. On each of day 3 and day 4 of the culturing, the culture supernatant was collected and filtered on a 0.2- μ m filter (Millipore) to remove cell debris therefrom. The purification of the humanized antibodies from the culture supernatant of the CHO cells was performed using POROS Protein A Column (PerSeptive Biosystems) on ConSep LC100 (Millipore) in accordance with the appended instructions. The humanized antibodies were provided for use in the determination of neutralizing activity and examination of pharmacological efficacy in hypercalcemic model animals. The concentration and the antigen-binding activity of the purified humanized antibodies were determined by the ELISA system as set forth above.

[REFERENCE EXAMPLE 5] Determination of neutralizing activity

The determination of neutralizing activity of the mouse antibodies, the chimeric antibodies and the humanized antibodies was performed using rat myeloma cell line ROS17/2.8-5 cells. The ROS17/2.8-5 cells were cultured in Ham'S F-12 medium (GIBCO) containing 10% fetal calf serum (GIBCO) in a CO_2 incubator. The ROS17/2.8-5 cells were seeded into each well of a 96-well plate at a density of 10^4 cells/100 μ l/well and cultured for one day. After the culturing was completed, the culture medium was replaced by Ham'S F-12 medium (GIBCO) containing 4 mM Hydrocortisone and 10% fetal calf serum. After culturing for three to four days, the cultured cells were washed with 260 μ l of Ham'S F-12 medium (GIBCO), and then added with 80 μ l of Ham's F-12 medium containing 1 mM isobutyl-1-methyl xanthine (IBMX, SIGMA), 10% fetal calf serum and 10 mM HEPES. The resulting mixture was incubated at 37° C for 30 min.

The culture mediums of the mouse antibodies, the chimeric antibodies and the humanized antibodies to be tested for neutralizing activity were previously diluted serially in the following dilution series: [10 μ g/ml, 3.3 μ g/ml, 1.1 μ g/ml and 0.37 μ g/ml], [10 μ g/ml,

2 μg/ml, 0.5 μg/ml and 0.01 μg/ml] and [10 μg/ml, 5 μg/ml, 1.25 μg/ml, 0.63 μg/ml and 0.31 μg/ml]. Each of the diluted antibody sample solutions was mixed with an equivalent amount of 4 ng/ml of PTHrP (1-34). The resulting mixed solution (80 μl) was added to each well. In each well, the final concentration of each antibody became a quarter of the above-mentioned concentration of the antibody, and accordingly the concentration of PTHrP (1-34) became 1 ng/ml. After the treatment at room temperature for 10 min., the culture supernatant was removed and the residue was washed with PBS three times. Subsequently, cAMP in the cells was extracted with 100 μl of a 0.3% HCl-95% ethanol and then evaporated using a water jet aspirator to remove the HCl-ethanol. The residue was dissolved in 120 μl of EIA buffer appended to cAMP EIA Kit (CAYMAN CHEMICAL'S) to extract the cAMP therefrom. The cAMP was determined using cAMP EIA Kit (CAYMAN CHEMICAL'S) in accordance with the instructions included in the kit. As a result, it was found that, among the humanized antibodies having the same levels of antigen-binding activity as that of the chimeric antibody, those antibodies having L-chain versions "q", "r", "s" and "t" (in which the 91-position tyrosine was replaced by isoleucine) exhibited the similar neutralizing activity to that of the chimeric antibody, and that antibody having a L-chain version "q" exhibited the strongest neutralizing activity.

<u>Sequence Listing Free Text</u>

SEQ ID NO: 1 Synthesized DNA

SEQ ID NO: 2 Synthesized DNA

SEQ ID NO: 3 Synthesized DNA

SEQ ID NO: 4 Synthesized DNA

SEQ ID NO: 5 Synthesized DNA

SEQ ID NO: 6 Synthesized DNA

SEQ ID NO: 7 Synthesized DNA

SEQ ID NO: 8 Synthesized DNA

SEQ ID NO: 9 Synthesized DNA

SEQ ID NO: 10 Synthesized DNA

SEQ ID NO: 11 Synthesized DNA

SEQ ID NO: 12 Synthesized DNA

SEQ ID NO: 13 Synthesized DNA

SEQ ID NO: 14 Synthesized DNA

SEQ ID NO: 15 Synthesized DNA

SEQ ID NO: 16 Synthesized DNA

SEQ ID NO: 17 Synthesized DNA

SEQ ID NO: 18 Synthesized DNA

SEQ ID NO: 19 Synthesized DNA

SEQ ID NO: 20 Synthesized DNA

SEQ ID NO: 21 Synthesized DNA

SEQ ID NO: 22 Synthesized DNA

SEQ ID NO: 23 Synthesized DNA

SEQ ID NO: 24 Synthesized DNA

SEQ ID NO: 25 Synthesized DNA

SEQ ID NO: 26 Synthesized DNA

SEQ ID NO: 27 Synthesized DNA

SEQ ID NO: 28 Synthesized DNA

SEQ ID NO: 29 Synthesized DNA

SEQ ID NO: 30 Synthesized DNA

SEQ ID NO: 31 Synthesized DNA

SEQ ID NO: 32 Synthesized DNA

SEQ ID NO: 33 Synthesized DNA

SEQ ID NO: 34 Synthesized DNA

SEQ ID NO: 35 Synthesized DNA

SEQ ID NO: 36 Synthesized DNA

SEQ ID NO: 37 Synthesized DNA

SEQ ID NO: 38 Synthesized DNA

SEQ ID NO: 39 Synthesized DNA

SEQ ID NO: 40 Synthesized DNA

SEQ ID NO: 41 Synthesized DNA

SEQ ID NO: 42 Synthesized DNA

SEQ ID NO: 43 Synthesized DNA

SEQ ID NO: 44 Synthesized DNA

All publications, patents and patent applications cited herein are incorporated by reference in their entirety.

INDUSTRIAL APPLICABILITY

As described above, the present invention provides a therapeutic agent for drug-resistant hypercalcemia, which comprises, as an active ingredient, a substance capable of inhibiting the binding between PTHrP and a receptor thereof.

According to the fact that when administered with the substance, the amelioration of blood calcium levels and body weights was observed in a model animal of drug-resistant hypercalcemia, it is found that the substance is useful as a therapeutic agent for drug-resistant hypercalcemia.

CLAIMS

1. A therapeutic agent for drug-resistant hypercalcemia comprising, as an active ingredient, a substance capable of inhibiting the binding between parathyroid hormone related protein (PTHrP) and a receptor thereof.

2. The therapeutic agent according to claim 1, wherein the drug-resistant hypercalcemia is resistant to a therapeutic agent for hypercalcemia other than a substance capable of inhibiting the binding between PTHrP and a receptor thereof.

3. The therapeutic agent according to claim 1 or 2, wherein the therapeutic agent for hypercalcemia is a bone resorption-inhibiting agent, a calcium excretion-promoting agent, an agent for inhibiting intestinal absorption of calcium or a loop diuretic.

4. The therapeutic agent according to claim 1 or 2, wherein the therapeutic agent for hypercalcemia is a bone resorption-inhibiting agent.

5. The therapeutic agent according to claim 4, wherein the bone resorption-inhibiting agent is a bisphosphonate or calcitonin.

6. The therapeutic agent according to any one of claims 1 to 5, wherein the substance is an antagonist for the PTHrP receptor.

7. The therapeutic agent according to any one of claims 1 to 5, wherein the substance is an anti-PTHrP antibody.

8. The therapeutic agent according to any one of claims 1 to 5, wherein the substance is a fragment of an anti-PTHrP antibody and/or a modified form of the fragment.

9. The therapeutic agent according to claim 7, wherein the antibody is monoclonal.

10. The therapeutic agent according to claim 7, wherein the antibody is a human antibody, or a humanized or chimeric antibody.

11. The therapeutic agent according to claim 7, wherein the antibody is in a humanized form.

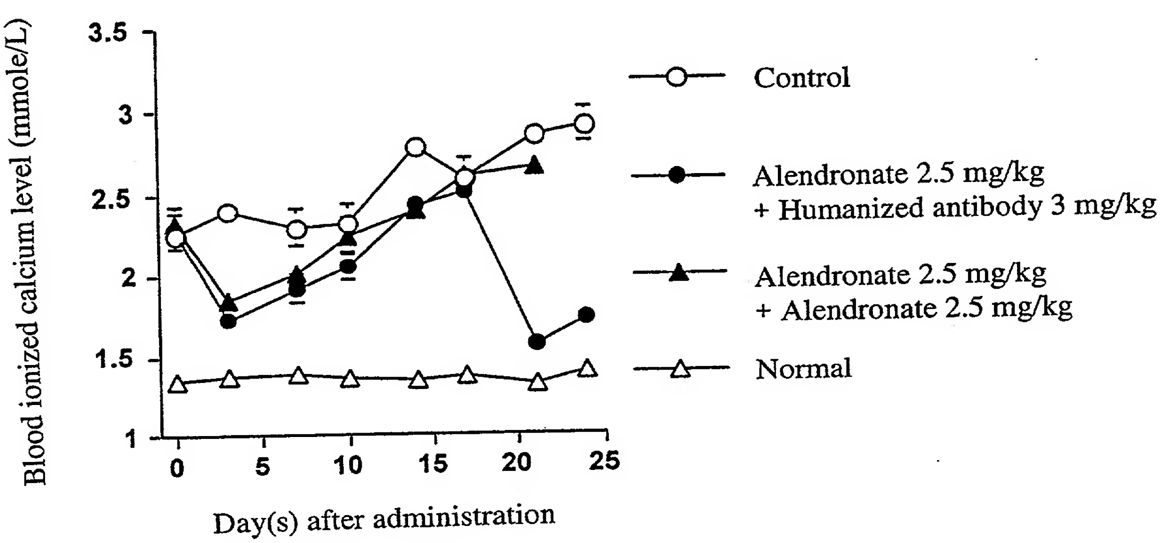
12. The therapeutic agent according to claim 11, wherein the humanized antibody is humanized #23-57-137-1 antibody.

13. The therapeutic agent according to any one of claims 1 to 12, wherein the drug-resistant hypercalcemia is caused by cancer.

ABSTRACT

The invention provides a therapeutic agent for drug-resistant hypercalcemia. The therapeutic agent comprises, as an active ingredient, a substance capable of inhibiting the binding between a parathyroid hormone related protein (PTHrP) and a receptor thereof.

Fig. 1



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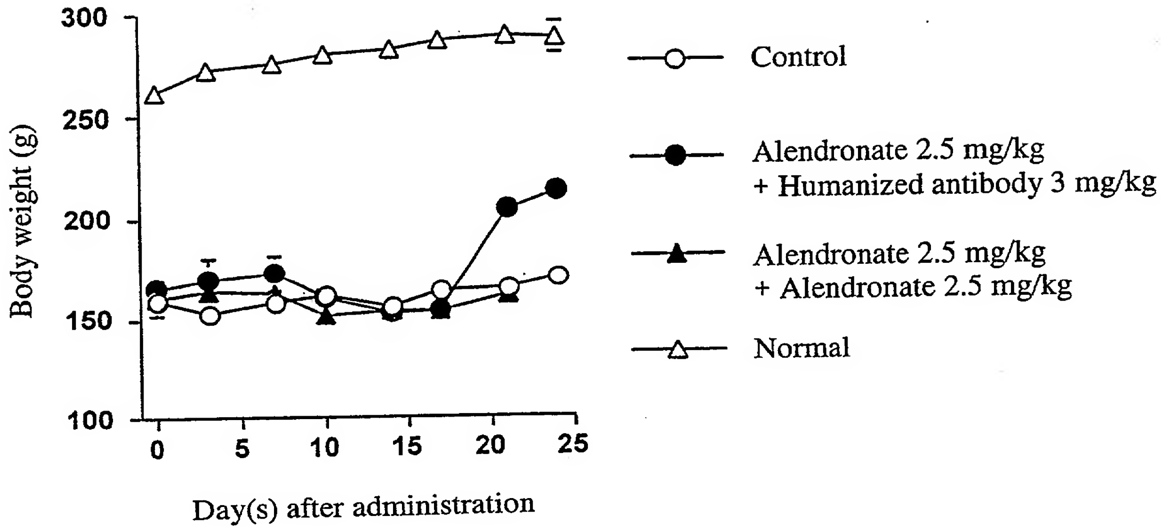

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200
150
100
Body weight (g)
0
5
10
15
20
25
Day(s) after administration
Control
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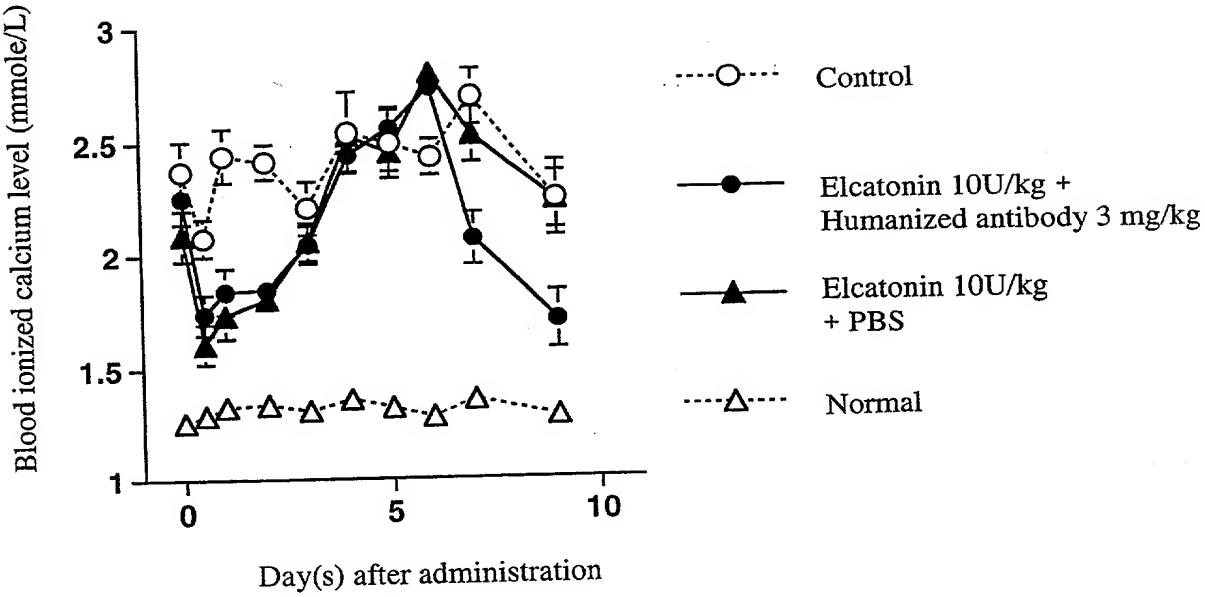
+ Humanized antibody 3 mg/kg
Alendronate 2.5 mg/kg
+ Alendronate 2.5 mg/kg
Normal

Fig. 3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2
1.5
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0
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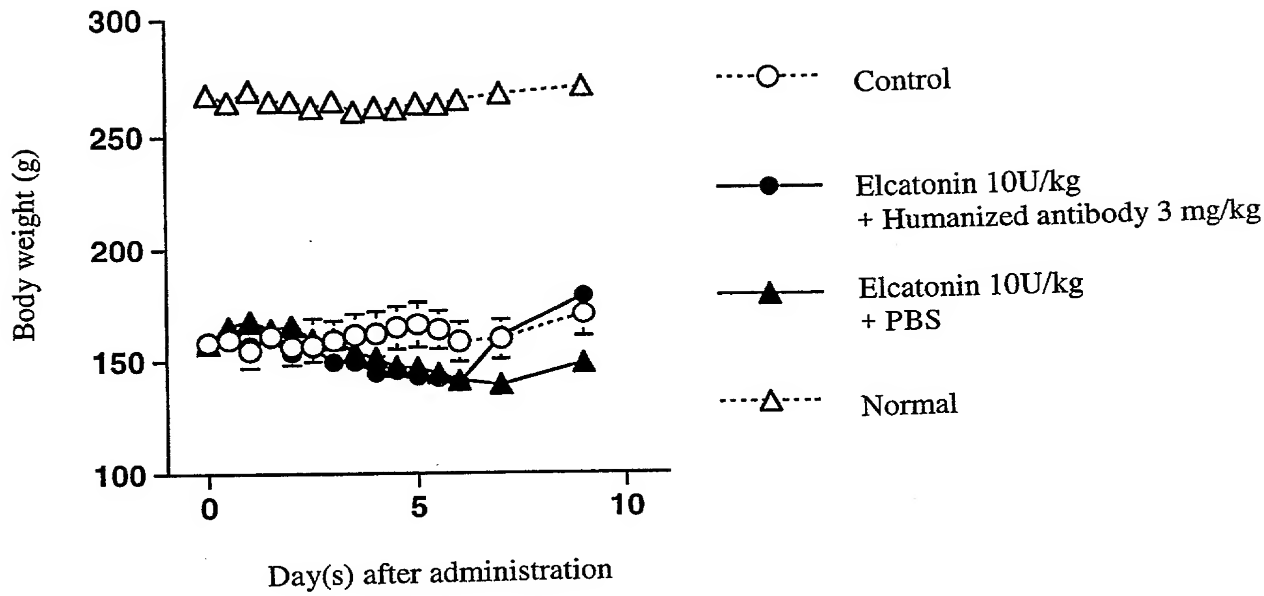
10
Day(s) after administration
Control
Elcatonin 10U/kg +
Humanized antibody 3 mg/kg
Elcatonin 10U/kg
+ PBS
Normal

Fig. 4



Attorney's Docket No.:_____

DECLARATION, POWER OF ATTORNEY AND PETITION

I (We), the undersigned inventor(s), hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I (We) believe that I am (we are) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

THERAPEUTIC AGENT FOR DRUG-RESISTANT HYPERCALCEMIA

the specification of which

☐ is attached hereto.

☐ was filed on _____ as Application Serial No. _____ and amended on _____

■ was filed as PCT international application Number PCT/JP00/04523 on July 6, 2000 and was amended under PCT Article 19 on _____ (if applicable).

I (We) hereby state that I (We) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; that I (We) do not know and do not believe that this invention was ever known or used before my invention or discovery thereof, or patented or described in any printed publication in any country before my invention or discovery thereof, or more than one year prior to this application, or in public use or on sale in the United States for more than one year prior to this application; that this invention or discovery has not been patented or made the subject of an inventor's certificate in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months before this application.

I (We) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

I (We) hereby claim foreign priority benefits under Section 119(a)-(d) of Title 35 United States Code, of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Application No.	Country	Filing date	Priority claimed
192270/1999	Japan	July 6, 1999	■ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

I hereby claim the benefit under Section 119(e) of Title 35 United States Code, of any United States application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

I (We) hereby claim the benefit under Section 120 of Title 35 United States Code, of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Section 112 of Title 35 United States Code, I (We) acknowledge the duty to disclose material information as defined in Section 1.56(a) of Title 37 Code of Federal Regulations, which occurred between the filing date of the prior application and national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented, abandoned)

And I (We) hereby appoint: FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P., CUSTOMER NUMBER 22,852

I(We) hereby request that all correspondence regarding this application be sent to the firm of FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P. whose Post office address is: 1300 I Street, N.W., WASHINGTON, D.C. 20005 U.S.A.

I (We) declare further that all statements made herein of my (our) knowledge are true and that all statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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Signature of Inventor

Date

Residence:

Citizen of:
Post Office Address:

SEQUENCE LISTING

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35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp

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Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp

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Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp

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20 25 30

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35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp

50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser

65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp

85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu

100 105 110

Thr Val Leu Gly Gln Pro

115

<210> 54

<211> 118

<212> PRT

<213> Homo sapiens

<400> 54

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
1 5 10 15

Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30

Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys Tyr Val Met
35 40 45

Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60

Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80

Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
85 90 95

Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
100 105 110

Thr Val Leu Gly Gln Pro
115

<210> 55

<211> 118

<212> PRT

<213> Homo sapiens

<400> 55

Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser Leu Gly Ala
1 5 10 15
Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser Thr Tyr Thr
20 25 30
Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg Tyr Val Met
35 40 45
Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly Ile Pro Asp
50 55 60
Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu Thr Ile Ser
65 70 75 80
Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly Val Gly Asp
85 90 95
Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly Thr Lys Leu
100 105 110
Thr Val Leu Gly Gln Pro
115

<210> 56
<211> 118
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<213> Homo sapiens

<400> 56
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1 5 10 15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30
Gly Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Ser Val

50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr

65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys

85 90 95

Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly Gln Gly Thr

100 105 110

Leu Val Thr Val Ser Ser

115

<210> 57

<211> 411

<212> DNA

<213> Mus musculus

<220>

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<222> (1)..(411)

<220>

<221> mat_peptide

<222> (58)..(411)

<400> 57

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Met Asn Phe Gly Leu Ser Leu Ile Phe Leu Ala Leu Ile Leu Lys Gly

-15 -10 -5

gtc cag tgt gag gtg caa ctg gtg gag tct ggg gga gac tta gtg aag 96

Val Gln Cys Glu Val Gln Leu Val Glu Ser Gly Gly Asp Leu Val Lys

-1 1 5 10

cct gga ggg tcc ctg aaa ctc tcc tgt gca gcc tct gga ttc act ttc 144

Pro Gly Gly Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe

15 20 25

agt agc tat ggc atg tct tgg att cgc cag act cca gac aag agg ctg 192

Ser Ser Tyr Gly Met Ser Trp Ile Arg Gln Thr Pro Asp Lys Arg Leu

30 35 40 45

gag tgg gtc gca acc att agt agt ggt ggt agt tac acc tac tat cca 240

Glu Trp Val Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro

50 55 60

gac agt gtg aag ggg cga ttc acc atc tcc aga gac aat gcc aag aac 288

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn

65 70 75

acc cta tac ctg caa atg agc agt ctg aag tct gag gac aca gcc atg 336

Thr Leu Tyr Leu Gln Met Ser Ser Leu Lys Ser Glu Asp Thr Ala Met

80 85 90

ttt tac tgt gca aga cag act act atg act tac ttt gct tac tgg ggc 384

Phe Tyr Cys Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly

95 100 105

caa ggg act ctg gtc act gtc tct gca 411

Gln Gly Thr Leu Val Thr Val Ser Ala

110 115

<210> 58

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<212> DNA

<213> Homo sapiens

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<221> mat_peptide

<222> (58)..(411)

<400> 58

atg ggg ttt ggg ctg agc tgg gtt ttc ctc gtt gct ctt tta aga ggt 48
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-15 -10 -5

gtc cag tgt cag gtg cag ctg gtg gag tct ggg gga ggc gtg gtc cag 96
Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln
-1 1 5 10

cct ggg agg tcc ctg aga ctc tcc tgt gca gcc tct gga ttc acc ttc 144
Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
15 20 25

agt agc tat ggc atg tct tgg gtc cgc cag gct cca ggc aag ggg ctg 192
Ser Ser Tyr Gly Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
30 35 40 45

gag tgg gtg gca acc att agt agt ggt ggt agt tac acc tac tat cca 240
Glu Trp Val Ala Thr Ile Ser Ser Gly Gly Ser Tyr Thr Tyr Tyr Pro
50 55 60

gac agt gtg aag ggg cga ttc acc atc tcc aga gac aat tcc aag aac 288
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn
65 70 75

acg ctg tat ctg caa atg aac agc ctg aga gct gag gac acg gct gtg 336
Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val

80 85 90

tat tac tgt gcg aga cag act act atg act tac ttt gct tac tgg ggc 384
Tyr Tyr Cys Ala Arg Gln Thr Thr Met Thr Tyr Phe Ala Tyr Trp Gly
95 100 105

cag gga acc ctg gtc acc gtc tcc tca 411
Gln Gly Thr Leu Val Thr Val Ser Ser
110 115

<210> 59
<211> 11
<212> PRT
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<400> 59
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1 5 10

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<400> 60
Ser Ala Ser Asn Arg Tyr Thr
1 5

<210> 61
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<212> PRT

<213> Homo sapiens

<400> 61

Gln Gln His Tyr Ser Thr Pro Phe Thr

1 5

<210> 62

<211> 5

<212> PRT

<213> Homo sapiens

<400> 62

Pro Tyr Trp Met Gln

1 5

<210> 63

<211> 16

<212> PRT

<213> Homo sapiens

<400> 63

Ser Ile Phe Gly Asp Gly Asp Thr Arg Tyr Ser Gln Lys Phe Lys Gly

1 5 10 15

<210> 64

<211> 11

<212> PRT

<213> Homo sapiens

<400> 64

Gly Leu Arg Arg Gly Gly Tyr Tyr Phe Asp Tyr
1 5 10

<210> 65

<211> 411

<212> DNA

<213> Mus musculus

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<222> (58)..(411)

<400> 65

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-15 -10 -5

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Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Ser Ser Ala Ser Phe Ser
-1 1 5 10

ctg gga gcc tca gca aaa ctc acg tgc acc ttg agt agt cag cac agt 144
Leu Gly Ala Ser Ala Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
15 20 25

acg tac acc att gaa tgg tat cag caa cag cca ctc aag cct cct aag 192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Leu Lys Pro Pro Lys

30 35 40 45

tat gtg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240

Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly

50 55 60

att cct gat cgc ttc tct gga tcc agc tct ggt gct gat cgc tac ctt 288

Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Asp Arg Tyr Leu

65 70 75

agc att tcc aac atc cag cca gaa gat gaa gca atg tac atc tgt ggt 336

Ser Ile Ser Asn Ile Gln Pro Glu Asp Glu Ala Met Tyr Ile Cys Gly

80 85 90

gtg ggt gat aca att aag gaa caa ttt gtg tat gtt ttc ggc ggt ggg 384

Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly

95 100 105

acc aag gtc act gtc cta ggt cag ccc 411

Thr Lys Val Thr Val Leu Gly Gln Pro

110 115

<210> 66

<211> 411

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

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<220>

<221> mat_peptide

<222> (58)..(411)

<400> 66

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-15 -10 -5

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Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
-1 1 5 10

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
15 20 25

acg tac acc att gaa tgg cat cag cag cag cca gag aag ggc cct cgg 192
Thr Tyr Thr Ile Glu Trp His Gln Gln Gln Pro Glu Lys Gly Pro Arg
30 35 40 45

tac ttg atg aaa ctt aag caa gat gga agc cac agc aca ggt gat ggg 240
Tyr Leu Met Lys Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
50 55 60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
65 70 75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt 336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly
80 85 90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
95 100 105

acc aaa ctg acc gtc cta ggt cag ccc 411
Thr Lys Leu Thr Val Leu Gly Gln Pro
110 115

<210> 67

<211> 411

<212> DNA

<213> Homo sapiens

<220>

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<222> (1)..(411)

<220>

<221> mat_peptide

<222> (58)..(411)

<400> 67

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-15 -10 -5

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Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
-1 1 5 10

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
15 20 25

acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct aag 192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys
30 35 40 45

tac ctg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240
Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly

50 55 60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
65 70 75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt 336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly
80 85 90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
95 100 105

acc aaa ctg acc gtc cta ggc cag ccc 411
Thr Lys Leu Thr Val Leu Gly Gln Pro
110 115

<210> 68
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<400> 68
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Met Ala Trp Thr Pro Leu Phe Phe Phe Phe Val Leu His Cys Ser Gly

-15 -10 -5

tct ttc tcc cag ctt gtg ctg act caa tcg ccc tct gcc tct gcc tcc 96

Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser

-1 1 5 10

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144

Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser

15 20 25

acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct aag 192

Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys

30 35 40 45

tac gtg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240

Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly

50 55 60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288

Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu

65 70 75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt 336

Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly

80 85 90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384

Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly

95 100 105

acc aaa ctg acc gtc cta ggc cag ccc 411

Thr Lys Leu Thr Val Leu Gly Gln Pro

110 115

<210> 69

<211> 411

<212> DNA

<213> Homo sapiens

<220>

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<220>

<221> mat_peptide

<222> (58)..(411)

<400> 69

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-15 -10 -5

tct ttc tcc cag ctt gtg ctg act caa tcg ccc tct gcc tct gcc tcc 96
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
-1 1 5 10

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
15 20 25

acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct agg 192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg
30 35 40 45

tac ctg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240
Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
50 55 60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu

65 70 75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt 336

Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly

80 85 90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384

Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly

95 100 105

acc aaa ctg acc gtc cta ggc cag ccc 411

Thr Lys Leu Thr Val Leu Gly Gln Pro

110 115

<210> 70

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<220>

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<400> 70

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-15 -10 -5

tct ttc tcc cag ctt gtg ctg act caa tcg ccc tct gcc tct gcc tcc 96

Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser

-1 1 5 10

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144

Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser

15 20 25

acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct agg 192

Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg

30 35 40 45

tac gtg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240

Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly

50 55 60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288

Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu

65 70 75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat tac tgt ggt 336

Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Tyr Cys Gly

80 85 90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384

Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly

95 100 105

acc aaa ctg acc gtc cta ggc cag ccc 411

Thr Lys Leu Thr Val Leu Gly Gln Pro

110 115

<210> 71

<211> 411

<212> DNA

<213> Homo sapiens

<220>

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<220>

<221> mat_peptide

<222> (58)..(411)

<400> 71

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                -15                 -10                 -5

tct ttc tcc cag ctt gtg ctg act caa tcg ccc tct gcc tct gcc tcc    96
Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
            -1   1               5                  10

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt    144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
        15                  20                  25

acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct aag    192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys
    30                  35                  40                  45

tac ctg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg    240
Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
                50                  55                  60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc    288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
            65                  70                  75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt    336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly
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80 85 90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
95 100 105

acc aaa ctg acc gtc cta ggc cag ccc 411
Thr Lys Leu Thr Val Leu Gly Gln Pro
110 115

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<220>
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Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
-1 1 5 10

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144

Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
15 20 25

acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct agg 192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg
30 35 40 45

tac ctg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240
Tyr Leu Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
50 55 60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
65 70 75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt 336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly
80 85 90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly
95 100 105

acc aaa ctg acc gtc cta ggc cag ccc 411
Thr Lys Leu Thr Val Leu Gly Gln Pro
110 115

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<400> 73

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Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser
-1 1 5 10

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144
Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser
15 20 25

acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct aag 192
Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Lys
30 35 40 45

tac gtg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240
Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly
50 55 60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288
Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu
65 70 75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt 336
Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly
80 85 90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384
Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly

95 100 105

acc aaa ctg acc gtc cta ggc cag ccc 411

Thr Lys Leu Thr Val Leu Gly Gln Pro

110 115

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<220>

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<221> mat_peptide

<222> (58)..(411)

<400> 74

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Ser Phe Ser Gln Leu Val Leu Thr Gln Ser Pro Ser Ala Ser Ala Ser

-1 1 5 10

ctg gga gcc tcg gtc aag ctc acc tgc acc ttg agt agt cag cac agt 144

Leu Gly Ala Ser Val Lys Leu Thr Cys Thr Leu Ser Ser Gln His Ser

15 20 25

acg tac acc att gaa tgg tat cag cag cag cca gag aag ggc cct agg 192

Thr Tyr Thr Ile Glu Trp Tyr Gln Gln Gln Pro Glu Lys Gly Pro Arg

30 35 40 45

tac gtg atg gat ctt aag caa gat gga agc cac agc aca ggt gat ggg 240

Tyr Val Met Asp Leu Lys Gln Asp Gly Ser His Ser Thr Gly Asp Gly

50 55 60

att cct gat cgc ttc tca ggc tcc agc tct ggg gct gag cgc tac ctc 288

Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser Gly Ala Glu Arg Tyr Leu

65 70 75

acc atc tcc agc ctc cag tct gag gat gag gct gac tat atc tgt ggt 336

Thr Ile Ser Ser Leu Gln Ser Glu Asp Glu Ala Asp Tyr Ile Cys Gly

80 85 90

gtg ggt gat aca att aag gaa caa ttt gtg tac gtg ttc ggc gga ggg 384

Val Gly Asp Thr Ile Lys Glu Gln Phe Val Tyr Val Phe Gly Gly Gly

95 100 105

acc aaa ctg acc gtc cta ggc cag ccc 411

Thr Lys Leu Thr Val Leu Gly Gln Pro

110 115

<210> 75

<211> 34

<212> PRT

<213> Homo sapiens

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Ala Val Ser Glu His Gln Leu Leu His Asp Lys Gly Lys Ser Ile Gln

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Asp Leu Arg Arg Arg Phe Phe Leu His His Leu Ile Ala Glu Ile His

20 25 30

Thr Ala